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(54) Title: MYCOBACTERIAL MUTANTS AFFECTING HOST APOPTOSIS

(57) Abstract: Provided are recombinant mycobacteria having a mutation in an*nlaA* gene or in a *nuoG* gene. Also provided are isolated and purified *nlaA* proteins and *nuoG* proteins from a mycobacterium. Additionally provided are isolated and purified nucleic acids comprising a recombinant *nlaA* gene or a recombinant *nuoG* gene. Further provided are methods of inducing an immune response in a mammal and methods of making a recombinant mycobacterium using the *nlaA* gene or the *nuoG* gene.

MYCOBACTERIAL MUTANTS AFFECTING HOST APOPTOSIS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 60/643,614, filed January 12, 2005.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grants No. R01 AI54540, AI26170, AI063537, and AI57158, all awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention generally relates to mutants of *Mycobacterium tuberculosis*. More particularly, the invention is directed to *M. tuberculosis* mutants that affect host cell apoptosis.

(2) Description of the Related Art

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Mycobacterium tuberculosis, the etiological agent of tuberculosis, is responsible for more deaths each year than any other single pathogen (Corbett *et al.*, 2003). The emergence of drug resistant strains of *M. tuberculosis* and HIV co-infection has contributed to the worsening impact of this disease. The pathogen exhibits extraordinary capacity to subvert and resist bactericidal responses of its infected host. *M. tuberculosis* virulence has been associated with its initial survival within macrophages by evading the host response in many different ways. The tubercle

bacilli reside in endocytic vacuoles (Armstrong and Hart, 1975; Clemens and Horwitz, 1995), which fail to fuse to lysosomes due to *M. tuberculosis* mediated retention of a host protein TACO on the membrane of these vacuoles (Gatfield and Pieters, 2000). Similarly, *M. tuberculosis* can downregulate the expression of MHC-II (Noss *et al.*, 2001) and costimulatory molecules (Stenger *et al.*, 1998; Wadee *et al.*, 1995), modulate the cytokine environment in its vicinity (VanHeyningen *et al.*, 1997) and inhibit apoptosis of the host cell (Keane *et al.*, 1997). Although *M. tuberculosis* evades many host responses to maintain itself in a habitable environment, the bacterial effectors mediating such effects need to be delineated. On invading the host cell, a capsule-like structure is formed outside the membrane and the cell wall of the tubercle bacilli (Daffe and Etienne, 1999), and this interface contains important surface proteins involved in the pathogenesis and immune responses to TB, The secreted and cell envelope associated proteins, located at the interface between the mycobacterium and its eukaryotic host mediate host-pathogen interactions. Therefore, such proteins are candidate virulence factors and warrants further study (Finlay and Falkow, 1997).

The exported and secreted proteins of *M. tuberculosis* have been proposed to play a role in virulence and indeed contribute to the immune responses to TB (Abou-Zeid *et al.*, 1988; Johansen *et al.*, 1996; Nagai *et al.*, 1991; Zhang *et al.*, 1992). Research on several bacterial pathogens has revealed that the majority of virulence factors are secreted (Finlay and Falkow, 1997). Studies have also emphasized the importance of the secreted and exported proteins of *M. tuberculosis* in the generation of a protective immune response. The most striking demonstration of this property comes from experiments in which mice or guinea pigs were immunized with extracellular proteins and significant protective immunity elicited (Andersen, 1994; Hubbard *et al.*, 1992; Pal and Horwitz, 1992; Roberts *et al.*, 1995). Recently, the exported ERP (exported repetitive protein) protein was shown to contribute to the virulence of *M. tuberculosis* (Berthet *et al.*, 1998). Likewise, superoxide dismutase (SOD), a culture filtrate component was shown to be associated with virulence by interfering with host apoptosis (Edwards *et al.*, 2001). While many secreted proteins have been studied, the study of the cell surface proteins is still lacking due to technological constraints in isolating samples of membrane proteins.

Host cell apoptosis has been implicated in *Mycobacterium spp.* virulence and protective immunity (e.g., Alemán et al., 2002; Balcewicz-Sablinska et al., 1998; Ciaramella et al., 2000; Duan et al., 2001, 2002; Duarte et al., 1997; Eddine et al., 2005; Grode et al., 2005; Keane et al., 2000; Kornfeld et al., 1999; López et al., 2003; Protales-Pérez et al., 2002; Sly et al., 2003; Spira et al., 2003). However, there is need for more information on *Mycobacterium* host genes that affect host cell apoptosis. The present invention addresses that need.

SUMMARY OF THE INVENTION

The present invention identifies mycobacterial genes that encode proteins that inhibit host apoptosis. Mycobacterium mutants that do not express the proteins are useful for inducing immunity to virulent mycobacteria.

Thus, the present invention is directed to recombinant mycobacteria having a mutation in an *nlaA* gene. The mutation in these mycobacteria increases the ability of the mycobacteria to induce apoptosis of a mammalian macrophage infected by the mycobacteria.

The invention is also directed to recombinant mycobacteria having a mutation in a *nuoG* gene. The mutation in these mycobacteria also increases the ability of the mycobacteria to induce apoptosis of a mammalian macrophage infected by the mycobacteria.

The present invention is additionally directed to isolated and purified nlaA proteins from a mycobacterium. These nlaA proteins have an amino acid sequence at least 85% identical to SEQ ID NO:1. These nlaA proteins prevent the mycobacterium from inducing apoptosis in a mammalian macrophage.

The invention is further directed to isolated and purified nuoG proteins from a mycobacterium. These nuoG proteins have an amino acid sequence at least 85% identical to SEQ ID NO:3. These nuoG proteins also prevent the mycobacterium from inducing apoptosis in a mammalian macrophage.

The present invention is also directed to isolated and purified nucleic acids comprising a recombinant *nlaA* gene having a nucleotide sequence at least 85% identical to SEQ ID NO:2.

Additionally, the invention is directed to isolated and purified nucleic acids comprising a recombinant *nuoG* gene having a nucleotide sequence at least 85% identical to SEQ ID NO:4.

The current invention is further directed to methods of inducing an immune response in a mammal. The methods comprise inoculating the mammal with any of the above-described mycobacteria.

The invention is additionally directed to methods of making a recombinant mycobacterium. The methods comprise eliminating expression of the *nlaA* gene in the mycobacterium.

The present invention is further directed to additional methods of making a recombinant mycobacterium. The methods comprise eliminating expression of the *nuoG* gene in the mycobacterium.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing that a deletion mutant induces more apoptosis than wild-type. Nuclear fragmentation was assayed by TUNEL followed by flow cytometric analysis from THP1

cells after 3, 5 and 7 days of infection with *M. tuberculosis* H37Rv, *M. tuberculosis ΔnlaA, M. bovis* BCG, *M. kansasii* and the complemented strain at an infectious dose of 10 bacilli per macrophage (MOI). Results are representative of three independent experiments done in triplicates.

FIG. 2 is graphs showing that the deletion mutant induces more apoptosis than wild-type which is not a result of limited growth *in vitro.* A) Growth of wild-type *M. tuberculosis* H37Rv and *M. tuberculosis ΔnlaA* in THP-1 macrophages at 3, 5 and 8 days post infection. B) Nuclear fragmentation assayed by TUNEL followed by flow cytometric analysis from THP1 cells after 3, 5 and 7 days of infection with *M. tuberculosis* H37Rv, *M. tuberculosis ΔnlaA* at an infectious dose of 10 bacilli per macrophage (MOI). Results are representative of three independent experiments done in triplicates.

FIG. 3 is graphs showing the deletion mutant induces more apoptosis than wild-type which is not a result of limited growth *in vitro.* Representative flow cytometric analysis of TUNEL stained apoptotic THP-1 cells following 3 days of infection with *M.tuberculosis* H37Rv, *nlaA* mutant and the complemented strain. Dotted line represent levels of apoptosis in the absence of any infection. Solid lines represent the levels of apoptosis under infection conditions.

FIG. 4 is graphs showing the deletion mutant induces more apoptosis than wild-type that is specific to the *nlaA* gene. Nuclear fragmentation assayed by TUNEL followed by flow cytometric analysis from THP1 cells after 3 of infection with *M. tuberculosis* H37Rv, *M. tuberculosis ΔnlaA* and the complemented strain at an MOI of 5 A) or B) MOI of 10 or C) *M. tuberculosis* H37Rv, *M. tuberculosis ΔnlaA, M. tuberculosis ΔpanCD* at an infectious dose of 10 bacilli per macrophage. Results are representative of three independent experiments done in triplicates. ** $p < 0.0001$ *$p < 0.002$ compared to wild-type H37Rv.

FIG. 5 is graphs showing the deletion mutant displays growth defects in the organs of infected mice. Total CFU counts of wild-type *M. tuberculosis* H37Rv (•), *M. tuberculosis ΔnlaA* (◊) and the complemented strain (▲) at various time points in lungs (A) and spleen (B) of mice infected via aerosol route. Data are expressed as log10 value of mean number of bacteria +/- standard deviation recovered from each mouse. Groups of four mice were evaluated at each time point. *** $p < 0.0001$ ** $p < 0.001$ *$p < 0.01$ compared to wild-type H37Rv.

FIG. 6 is graphs showing the deletion mutant displays growth defects in the organs of infected mice. Total CFU counts of wild-type *M. tuberculosis* H37Rv (•), *M. tuberculosis ΔnlaA* (◊) and the complemented strain (▲) at various time points in lungs (A) and spleen (B) of mice infected intravenously (a & b). Data are expressed as log10 value of mean number of bacteria +/- standard deviation recovered from each mouse. Groups of four mice were evaluated at each time point. *$p < 0.01$ compared to wild-type H37Rv

FIG. 7 is a graph showing the deletion mutant is attenuated in mice. Time-to-death analysis in C57Bl/6 mice upon aerosol infection with wild-type *M. tuberculosis* H37Rv (•), *M. tuberculosis ΔnlaA* (◊) and the complemented strain (▲) shows significant mortality delay. The inoculum was 100 cfu for aerosol infection (n = 10). $p < 0.0001$ compared to wild-type H37Rv and complemented strain.

FIG. 8 is a graph showing the deletion mutant is attenuated in mice. Time-to-death analysis in C57Bl/6 mice upon intravenous infection with wild-type *M. tuberculosis* H37Rv (•), *M. tuberculosis ΔnlaA* (◊) and the complemented strain (▲) shows significant mortality delay. The inoculum was 10^6 cfu (n = 10). $p<0.0001$ compared to wild-type H37Rv.

FIG. 9 is a graph demonstrating that SCID mice display similar mortality rates with the wild-type strain and the deletion mutant. The inoculum was 10^4 cfu (n = 10).

FIG. 10 is graphs showing that the deletion mutant displays more apoptotic cells in the organs of infected mice. Percentage apoptotic cells as determined by review of multiple 40x magnification fields following staining with TUNEL in the spleens (A) and lungs (B) of mice infected with 10^6 cfu of the *M. tuberculosis* Δ*nlaA* (filled bars) and the virulent wild-type *M. tuberculosis* H37Rv (empty bars). Nuclei of cells undergoing apoptosis stain brown with TUNEL.

FIGS. 11 and 12 are micrographs of stained cells showing the deletion mutant induces increased levels of apoptosis *in vivo*. Representative TUNEL-stained views of spleen tissue sections (FIG. 11) and lung tissue sections (FIG. 12) harvested at 1 week and 3 weeks following intravenous infection with 10^6 cfu of *M. tuberculosis* Δ*nlaA* and the wild-type *M. tuberculosis* H37Rv photographed at 40x. Nuclei of cells undergoing apoptosis stain brown with TUNEL.

FIGS. 13 to 16 are micrographs showing the deletion mutant produces less tissue pathology in the lungs of infected mice compared with wild-type *M. tuberculosis* H37Rv. C57BL/6 mice infected with 100 CFU by aerosol route were evaluated at 3 weeks (Figure 13), 8 weeks (Figure 14), 12 weeks (Figure15), and 21 weeks (Figure 16) at 2.5x (A, B) and 40x (C, D) following hematoxylin and eosin staining of lung tissues.

FIG. 17 is a graph showing the deletion mutant produces less tissue pathology in the liver of infected mice compared with wild-type *M. tuberculosis* H37Rv. Graphical representation of the number of granulomas as determined from review of the whole liver following intravenous infection of C57BL/6 mice with *M. tuberculosis* Δ*nlaA* or wild type *M. tuberculosis*. Four sections from each mouse were reviewed at 10x magnification and groups of three mice and two sections per mouse were evaluated for each time point.

FIG. 18 is a graph of the CD4+ cells reactive against ESAT-6 in mice infected with Δ*nlaA* or wild type *M. tuberculosis*. Normal C57BL/6 mice were infected by the intravenous

route with 10^6 colony forming units of either *Mycobacterium tuberculosis* H37Rv or the Δ*nlaA* mutant of H37Rv. Four mice were infected with each strain, and an additional four mice were left uninfected as controls. After 4 weeks, the animals were sacrificed, their spleens removed and used to prepare single cell suspensions. Cultured splenocytes from each mouse were stimulated in triplicate cultures with 100 micrograms/ml of purified recombinant *M. tuberculosis* ESAT-6 protein antigen, and after 24 hours were analyzed by interferon-γ (IFNγ) ELISPOT. Results show significant induction of ESAT-6 reactive IFNγ-producing T cells in mice infected with both strains compared to control uninfected mice. Most notably, there were markedly elevated levels in three of the four Δ*nlaA* infected mice compared to mice infected with wild type H37Rv. Each bar represents one mouse. Note that this assay detects almost exclusively CD4+ T cells.

FIG. 19 is the same as FIG 18, except that splenocytes were stimulated with 100 micrograms/ml of purified recombinant *M. tuberculosis* Ag85A antigen. As with the ESAT-6 response, significant numbers of IFNγ producing T cells were detected against this antigen in all infected mice, and the numbers were significantly greater for the animals infected with the Δ*nlaA* strain.

FIG. 20 is micrographs and graphs of experimental results identifying three independent regions in the *M. tuberculosis* genome that mediate the inhibition of macrophage apoptosis. Panel a. *M. smegmatis* induced more cell death in infected THP-1 cells than BCG as observed by bright field microscopy (left panels). Fluorescence microscopy showed TUNEL staining in *M. smegmatis* infected cells undergoing apoptosis (Middle panels; red fluorescence is TUNEL staining, and green fluorescence is GFP-labeled bacteria. The light areas in the center micrograph is almost entirely green; the light areas in the bottom micrograph are almost entirely red). Strong induction of apoptosis by *M. smegmatis* was confirmed by flow cytometry (right panels; Y-axis shows TUNEL staining, X-axis is forward light scatter which reveals marked reduction in cell size associated with massive cell death in the *M. smegmatis*-infected cultures). Panel b. *M. smegmatis* was transfected with an episomal cosmid library of *M. tuberculosis* genomic DNA and individual clones were screened for their capacity to inhibit apoptosis. The final screen was performed by TUNEL staining and flow cytometry. Untreated (UT) cells and *M. smegmatis* (Msm) and BCG infected THP-1 cells were compared to THP-1 cells infected with *M. smegmatis* carrying the indicated *M. tuberculosis* cosmid clones. Panel c. The cosmid DNA of three clones (J21, K20, M24) was purified and used to re-transfect M. smegmatis, resulting in clones J21-b, K20-b and M24-b. These clones along with the original transformants were tested for induction of apoptosis by infection of THP-1 cells and analyzed as in Panel b. Panel d. The three selected cosmid DNAs and empty vector cosmid (CO) were transfected into *M. kansasii* (Mkan), and the induction of apoptosis by the bacteria was compared to untreated (UT) and *M. tuberculosis* (Mtb)

infected THP-1 cells using TUNEL assay at day 5 day of infection as in Panel b. $p<0.01$ for J21, M24, and K20 compared to CO by unpaired t-test. Results shown in Panels a through d are all representative of at least three independent experiments.

FIG. 21 is graphs and micrographs showing the correlation between apoptosis inhibition and mycobacterial virulence. SCID mice were infected with 10^6 bacteria via the tail vain and survival (Panel a, seven mice per group), or the bacterial load in lung, liver and spleen was determined (Panel b, three mice per group). *M. tuberculosis* (squares), Mkan-J21 (triangle), Mkan-M24 (inverted triangle), Mkan-CO (circle) and Mkan-K20 (diamonds). Panel c, Lung histopathology of infected mice was analyzed after 2 and 5 weeks of infection. Panel d, Levels of apoptosis in the lungs after 2 weeks of infection were determined by TUNEL staining of tissue sections. Panels c and d show representative fields for infections with each bacterial strain as indicated. The graph in panel d shows quantitation of TUNEL positive cells, and asterisks indicate statistically significant reductions ($p < 0.005$, unpaired t-test) compared to Mkan-CO infected mice. The results shown are representative of three independent experiments.

FIG. 22 is graphs identifying *nuoG* as a mycobacterial virulence determinant. Selected genes of the cosmid J21 were deleted in *M. tuberculosis* by specialized transduction and the resulting strains were analyzed for their virulence in SCID mice (Panel a) and for their capacity to inhibit infection-induced apoptosis in human macrophages (Panel b). The Δ*nuoG* bacteria were complemented with *nuoG*. The virulence of the wild-type (square), Δ*nuoG* (triangle) and complemented (open triangle) *M. tuberculosis* strains was compared in SCID mice (Panel c). Results shown are representative of two experiments for Panels a and c and three for (panel b).

FIG. 23 is a graphic summary and a photograph of an ethidium bromide-stained gel illustrating the gain-of-function screen described in Example 2. The top panel summarizes the results from the gain-of-function screen. The bottom panel shows a restriction digest analysis of cosmid DNA of 9 selected clones. The final four selected clones are I16, J21, K20 and M24. Only the 8 kbp side band in the M6 lane is specific for M6; the other bands are spillover from the M24 lane.

FIG. 24 is a diagram of the insert of cosmid J21. The diagram and legend was created using the TubercuList web server. The position of deletion mutants used to identify the gene that mediates the inhibition of infection-induced apoptosis are indicated. Arrows indicate single gene deletions and the large deletion from PPE53 to Rv3163c is marked.

FIG. 25 is a diagram and a photograph of a Southern blot illustrating the creation of a *nuoG* null mutant in *M. tuberculosis*. The hygromycin cassette was introduced into the genome via specialized transduction. The insertion was confirmed by Southern blot using the radiolabeled probe hybridizing at the indicated position.

FIG. 26 is graphs of forward light scattering and TUNEL assays showing induction of apoptosis in THP-1 cells by *M. tuberculosis* Δ*nlaA*.

FIG. 27 is graphs of forward light scattering and TUNEL assays showing induction of apoptosis in THP-1 cells by *M. tuberculosis* Δ*nlaA* and Δ*secA2* cells.

FIG. 28 is a diagram and graphs of TUNEL assays showing that restoring SodA secretion by a Δ*secA2* mutant reverses the effect of the *secA2* deletion on host cell apoptosis. Panel A is a diagram of the strategy for restoring SodA secretion in a Δ*secA2 M. tuberculosis* by expression of a transgenic *SodA* gene. Panel B is graphs of TUNEL assays showing induction of host cell apoptosis by Δ*secA2 M. tuberculosis* but not by Δ*secA2*-αSodA *M. tuberculosis*.

FIG. 29 is a diagram of the strategy used to evaluate the ability of various transgenic mycobacteria to induce interferon-γ (IFNγ) from OT-1 cells after exposure to macrophages. The mycobacteria are transfected with fusion proteins of *M. tuberculosis* antigens and the ovalbumin antigen SIINFEKL. Fusion proteins from transgenic mycobacteria that are apoptotic will be presented on the macrophage, where the SIINFEKL epitope will be recognized by the OT-1 cells, inducing IFNγ production by the OT-1 cells.

FIG. 30 is a graph of experimental data showing *in vitro* cross-presentation of the SIINFEKL peptide (OVA) by infected bone marrow-derived macrophages. The IFNγ production by the Δ*nlaA*-19k-OVA and Δ*SecA2*-19k-OVA indicates that the Δ*nlaA* and Δ*SecA2* mutants are apoptotic.

FIG. 31 is a graph of experimental data showing *in vivo* CD8 T cell priming by *M. tuberculosis* OVA (SIINFEKL)-expressing mutants with specific IFNγ ELISPOT analysis of splenocytes harvested on day 7 after i.v. mouse infection. The IFNγ production by the Δ*nlaA*-19k-OVA and Δ*SecA2*-19k-OVA indicates that the Δ*nlaA* and Δ*SecA2* mutants are apoptotic.

FIG. 32 is a diagram showing the strategy for determining the effect of infection with a mycobacterium on proliferation of carboxy-fluorescein diacetate, succinimidyl ester (CFSE)-labeled OT-1 splenocytes.

FIG. 33 is graphs of experimental results showing the activation of naïve $CD8^+$ T cells by apoptosis-inducing *M. tuberculosis* mutants Δ*nlaA*-19k-OVA and Δ*SecA2*-19k-OVA.

FIG. 34 is a diagram showing the strategy for determining the ability of a mycobacterium to induce a CTL immune response *in vivo*.

FIG. 35 is graphs of experimental results showing that *M. tuberculosis* mutants Δ*nlaA*-19k-OVA and Δ*SecA2*-19k-OVA induces a CTL response to cells presenting the 19k-OVA antigen.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have identified and characterized mycobacterium genes that prevent apoptosis of a mammalian cell infected by the mycobacterium. This finding enables and makes useful various compositions and methods relating to the use of recombinant mycobacteria deleted in the gene. Such mycobacteria are useful, for example, as live mycobacterial vaccines because the increased apoptosis of infected cells, particularly macrophages, allows for better presentation of antigens and induction of immunity. See Examples below.

Thus, the present invention is directed to recombinant mycobacteria having a mutation in an *nlaA* gene. The mutation in these mycobacteria increases the ability of the mycobacteria to induce apoptosis of a mammalian macrophage infected by the mycobacteria.

The amino acid and cDNA sequences for one form of the nlaA gene, from an *M. tuberculosis*, is provided herein as SEQ ID NO:1 and SEQ ID NO:2, respectively. However, these embodiments are not limited to the protein and gene provided as SEQ ID NO:1 and SEQ ID NO:2, since other mycobacteria would be expected to have forms of nlaA that have a different amino acid and protein sequence. The present invention would therefore encompass any nlaA protein and gene from a mycobacterium, which would be expected to be at least about 85%, or at least 95%, or at least 99% identical to SEQ ID NO:1 and SEQ ID NO:2, respectively. Any such form of nlaA could be identified and isolated without undue experimentation by a skilled artisan. Thus, the *nlaA* gene without the mutation preferably encodes a protein that is at least about 85% homologous to SEQ ID NO:1; more preferably, the *nlaA* gene without the mutation encodes a protein that is at least about 99% homologous to SEQ ID NO:1. Most preferably, the *nlaA* gene without the mutation encodes a protein having the amino acid sequence of SEQ ID NO:1.

Since these mycobacteria are designed to be used *in vivo*, it is preferred that the mycobacteria is avirulent or rendered so, e.g., by selecting for avirulent strains or by engineering the mycobacteria to have a mutation or mutations that can fulfill that purpose. Many such mutations are known in the art, for example mutations that render the mycobacterium auxotrophic, e.g., a *pan* mutation or a *Lys* mutation, or mutations eliminating pathogenicity genes such as an *RD1* deletion, as is known in the art. It is also preferred that the mycobacterium utilized for this invention can colonize the host, in order for the mycobacterium to provide a long term antigenic stimulus to the host, thus establishing a strong immune response. Non-limiting examples of useful mycobacteria are *Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG, *Mycobacterium avium*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium habana*, *Mycobacterium microti*, *Mycobacterium scrofulacium*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis*, and any genetic variant thereof. In some preferred embodiments the mycobacterium is a

Mycobacterium tuberculosis, since the *nlaA* gene, and the Δ*nlaA* mutation, is characterized herein in *M. tuberculosis*. Another particularly useful mycobacterial strain to incorporate a Δ*nlaA* mutation is *M. bovis* BCG.

Preferably, the mycobacterium further comprises a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell. Preferably, the gene encodes an antigen, for example to a neoplasm, tumor or cancer, or to a human pathogen, to take advantage of the increased immunogenicity to the antigen as a result of the Δ*nlaA* mutation. Examples of pathogens (e.g., human pathogens) where antigens useful in these mycobacteria include viruses (e.g., HIV, hepatitis C virus, herpes virus, influenza, smallpox, diphtheria, tetanus, measles, mumps, rabies, poliovirus etc), bacteria (e.g., pathogenic mycobacteria, *Salmonella* sp., etc.), and eukaryotic parasites (e.g., malaria, Leishmania, etc.).

The invention is also directed to recombinant mycobacteria having a mutation in a *nuoG* gene. The mutation in these mycobacteria also increases the ability of the mycobacteria to induce apoptosis of a mammalian macrophage infected by the mycobacteria. See Example 2.

The amino acid and cDNA sequences for one form of the *nuoG* gene, from an *M. tuberculosis*, is provided herein as SEQ ID NO:3 and SEQ ID NO:4, respectively. However, these embodiments are not limited to the protein and gene provided as SEQ ID NO:3 and SEQ ID NO:4, since other mycobacteria would be expected to have forms of nuoG that have a different amino acid and protein sequence. The present invention would therefore encompass any nuoG protein and gene from a mycobacterium, which would be expected to be at least about 85%, or at least 95%, or at least 99% identical to SEQ ID NO:3 and SEQ ID NO:4, respectively. Any such form of nuoG could be identified and isolated without undue experimentation by a skilled artisan. Thus, the *nuoG* gene without the mutation preferably encodes a protein that is at least about 85% homologous to SEQ ID NO:3; more preferably, the *nlaA* gene without the mutation encodes a protein that is at least about 99% homologous to SEQ ID NO:3. Most preferably, the *nuoG* gene without the mutation encodes a protein having the amino acid sequence of SEQ ID NO:3.

Since these mycobacteria are designed to be used *in vivo*, it is preferred that the mycobacteria is avirulent or rendered so, e.g., by selecting for avirulent strains or by engineering the mycobacteria to have a mutation or mutations that can fulfill that purpose. Many such mutations are known in the art, for example mutations that render the mycobacterium auxotrophic, e.g., a *pan* mutation or a *Lys* mutation, or mutations eliminating pathogenicity genes such as an *RD1* deletion, as is known in the art. It is also preferred that the mycobacterium utilized for this invention can colonize the host, in order for the mycobacterium to provide a long term antigenic stimulus to the host, thus establishing a strong immune response. Non-limiting examples of useful mycobacteria are *Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG,

Mycobacterium avium, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium habana*, *Mycobacterium microti*, *Mycobacterium scrofulacium*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis*, and any genetic variant thereof. In some preferred embodiments the mycobacterium is a *Mycobacterium tuberculosis*, since the *nuoG* gene, and the Δ*nuoG* mutation, is characterized herein in *M. tuberculosis*. Another particularly useful mycobacterial strain to incorporate a Δ*nuoG* mutation is *M. bovis* BCG.

Preferably, the mycobacteria further comprises a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell. Preferably, the gene encodes an antigen, for example to a neoplasm, tumor or cancer, or to a human pathogen, to take advantage of the increased immunogenicity to the antigen as a result of the Δ*nlaA* or Δ*nuoG* mutation. Examples of pathogens where antigens useful in these mycobacteria include viruses, bacteria, and eukaryotic parasites.

The present invention is additionally directed to isolated and purified nlaA proteins from a mycobacterium. These nlaA proteins have an amino acid sequence at least 85% identical to SEQ ID NO:1. These nlaA proteins prevent the mycobacterium from inducing apoptosis in a mammalian macrophage. The nlaA protein of these embodiments could be from any mycobacteria. Any mycobacterial protein having at least 85% identity to SEQ ID NO:1 would be expected to be an nlaA protein and would be expected to prevent apoptosis in a mammalian cell infected by a mycobacterium expressing that nlaA protein. Preferably, the amino acid sequence is at least 95% identical to SEQ ID NO:1; more preferably 99% identical to SEQ ID NO:1. In the most preferred embodiments, the amino acid sequence of the isolated protein is SEQ ID NO:1. Preferably, the nlaA protein was expressed recombinantly.

The invention is further directed to isolated and purified nuoG proteins from a mycobacterium. These nuoG proteins have an amino acid sequence at least 85% identical to SEQ ID NO:3. These nuoG proteins also prevent the mycobacterium from inducing apoptosis in a mammalian macrophage. The nuoG protein of these embodiments could be from any mycobacteria. Any mycobacterial protein having at least 85% identity to SEQ ID NO:3 would be expected to be an nuoG protein and would be expected to prevent apoptosis in a mammalian cell infected by a mycobacterium expressing that nlaA protein. Preferably, the amino acid sequence is at least 95% identical to SEQ ID NO:3; more preferably 99% identical to SEQ ID NO:3. In the most preferred embodiments, the amino acid sequence of the isolated protein is SEQ ID NO:3. Preferably, the nuoG protein was expressed recombinantly.

The present invention is also directed to isolated and purified nucleic acids comprising a recombinant *nlaA* gene having a nucleotide sequence at least 85% identical to SEQ ID NO:2.

Useful manipulations of the *nlaA* gene are provided in Example 1. In preferred embodiments, the nucleotide sequence is at least 95% identical to SEQ ID NO:2; in more preferred embodiments, the nucleotide sequence is at least 99% identical to SEQ ID NO:2; in the most preferred embodiments, the nucleotide sequence is SEQ ID NO:2.

Preferably, the nucleic acid is a vector capable of replication and/or expression of the nlaA protein encoded by the recombinant *nlaA* gene when transfected into a mycobacterium.

Additionally, the invention is directed to isolated and purified nucleic acids comprising a recombinant *nuoG* gene having a nucleotide sequence at least 85% identical to SEQ ID NO:4.

Useful manipulations of the *nuoG* gene are provided in Example 2. In preferred embodiments, the nucleotide sequence is at least 95% identical to SEQ ID NO:4; in more preferred embodiments, the nucleotide sequence is at least 99% identical to SEQ ID NO:4; in the most preferred embodiments, the nucleotide sequence is SEQ ID NO:4.

Preferably, the nucleic acid is a vector capable of replication and/or expression of the nlaA protein encoded by the recombinant *nuoG* gene when transfected into a mycobacterium.

The current invention is further directed to methods of inducing an immune response in a mammal. The methods comprise inoculating the mammal with any of the above-described mycobacteria. Preferably, the mycobacteria comprises a mutation in an *nlaA* gene or an *nuoG* gene, where the mutation increases the ability of the mycobacteria to induce apoptosis of a mammalian macrophage infected by the mycobacteria.

The mycobacteria utilized in these methods can also comprise a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell. Preferably, the gene encodes an antigen, for example to a neoplasm, tumor or cancer, or to a human pathogen, to take advantage of the increased immunogenicity to the antigen as a result of the Δ*nlaA* or Δ*nuoG* mutation. Examples of pathogens where antigens useful in these mycobacteria include viruses, bacteria, and eukaryotic parasites.

Preferably, the mycobacteria used in these methods is is an *M. tuberculosis* or an *M. bovis*. Also preferably, the inoculation given in as part of these methods gives the mammal increased immunity to a virulent *M. tuberculosis*.

The invention is additionally directed to methods of making a recombinant mycobacterium. The methods comprise eliminating expression of the *nlaA* gene in the mycobacterium. In preferred embodiments, expression of the *nlaA* gene is eliminated by specialized transduction, as is known in the art. A second gene can also be eliminated in these mycobacterium, wherein the mycobacterium exhibits attenuated virulence in a mammal when compared to the same mycobacterium expressing the second gene. A preferred second genes here is a portion of an RD1 region, or a gene controlling production of a vitamin or an amino acid.

Other preferred second genes that can usefully be eliminated here are those where eliminating expression of the second gene increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium..

These embodiments can be utilized with any species of mycobacterium. Preferred are avirulent species, or attenuated variants of a virulent species (e.g., *M. tuberculosis*, with, e.g., an RD1 or *pan* mutation). Nonlimiting examples of useful mycobacteria for this purpose are *Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG, *Mycobacterium avium*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium habana*, *Mycobacterium microti*, *Mycobacterium scrofulaceum*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis*, or any genetic variant thereof.

Analogous to previous embodiments, the mycobacterium preferably further comprises a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell. The recombinant gene in these embodiments preferably encodes an antigen of a cancer or a human pathogen, such as a virus, bacterium, or eukaryotic parasite, as discussed above.

The present invention is further directed to additional methods of making a recombinant mycobacterium. The methods comprise eliminating expression of the *nuoG* gene in the mycobacterium. In preferred embodiments, expression of the *nuoG* gene is eliminated by specialized transduction. A second gene can also be eliminated in these mycobacterium, wherein the mycobacterium exhibits attenuated virulence in a mammal when compared to the same mycobacterium expressing the second gene. A preferred second genes here is a portion of an RD1 region, or a gene controlling production of a vitamin or an amino acid. Other preferred second genes that can usefully be eliminated here are those where eliminating expression of the second gene increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium.

These embodiments can be utilized with any species of mycobacterium. Preferred are avirulent species, or attenuated variants of a virulent species (e.g., *M. tuberculosis*, with, e.g., an RD1 or *pan* mutation). Nonlimiting examples of useful mycobacteria for this purpose are *Mycobacterium smegmatis*, *Mycobacterium bovis*-BCG, *Mycobacterium avium*, *Mycobacterium phlei*, *Mycobacterium fortuitum*, *Mycobacterium lufu*, *Mycobacterium paratuberculosis*, *Mycobacterium habana*, *Mycobacterium microti*, *Mycobacterium scrofulaceum*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis*, or any genetic variant thereof.

Analogous to previous embodiments, the mycobacterium preferably further comprises a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell. The recombinant gene in these embodiments

preferably encodes an antigen of a cancer or a human pathogen, such as a virus, bacterium, or eukaryotic parasite, as discussed above.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims, which follow the examples.

Example 1. A newly identified protein of *Mycobacterium tuberculosis* evades host responses by inhibiting apoptosis.

Example summary

Mycobacterium tuberculosis is one of most successful pathogens of mankind, adapted for intracellular lifestyle and has extraordinary capacity to subvert host responses. This study describes the role of a newly identified exported protein, NlaA in promoting intracellular survival by evading host cell apoptosis. Deletion of this gene in *M. tuberculosis* resulted in a strain defective in inhibiting apoptosis of human macrophages and in the tissues of infected mice thereby resulting in its attenuation, growth defect and ability to cause less tissue damage.

On the assumption that *M. tuberculosis* secretes or exports proteins that interact with macrophage proteins or non-protein products to enhance its survival in a mammalian host, we undertook a screen for surface associated or secreted proteins using a reporter *phoA* technology developed earlier (Braunstein *et al.*, 2000). The *phoA* (*Escherichia coli* alkaline phosphatase) reporter technology has been used successfully in identifying secreted and exported proteins of bacteria (Carroll *et al.*, 2000; Lim *et al.*, 1995). A *phoA* gene that lacks signals for expression and export is active only when it is located outside of the cytoplasm; therefore, enzymatically active PhoA fusion proteins identify exported proteins. In this report, we describe the role of a novel gene involving the ORF *Rv3238c*, the hypothetical product of which is homologous to the human nuclear rim protein, Nurim (Rolls *et al.*, 1999), and to Isoprenyl-cysteine-carboxy-methyl-transferase (ICMT) enzyme of many bacteria. Although, the role of ICMT in these bacteria is unknown, it has been shown that inhibition of this enzyme in human pulmonary artery endothelial cell (PAEC) induces apoptosis of these cells, but its overexpression protects against apoptosis (Kramer *et al.*, 2003). This association prompted us to disrupt the *Rv3238c* gene in *M. tuberculosis* and characterize the resulting mutant in various models of tuberculosis. We hypothesized that *Rv3238c* is a bacterial effector molecule that enhances survival of *M. tuberculosis* in a mammalian host by preventing macrophage apoptosis.

Our studies demonstrate that the *Rv3238c* mutant of *M. tuberculosis* is defective in inhibiting apoptosis of human macrophages and in the tissues of infected mice thereby resulting in its attenuation, growth defect and ability to cause less tissue damage in immunocompetent C57/Bl6 mice. We have thus designated *Rv3238c* as *nlaA* (Nurim-like anti-apoptotic) gene.

Materials And Methods

Mycobacteria cultures. Cultures of wild-type *Mycobacterium bovis*, *M. bovis* bacillus Calmette-Guérin (BCG), *M. bovis* bacillus Calmette-Guérin (BCG) Δ*nlaA*, *M. kansasi*, *M. tuberculosis*, *M. tuberculosis* Δ*nlaA*, *M. tuberculosis* Δ*nlaA* complemented strain were grown to logarithmic phase in Middlebrook 7H9 broth (GIBCO).

THP-1 cells. THP-1 cells (American Type Culture Collection, Manassas, Va.) were grown in RPMI 1640 (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), 1% HEPES. The cells were treated with 5 nM phorbol myristate acetate (PMA; Sigma) overnight and then washed three times before infecting them with bacterial strains.

Infection of THP-1 cells. Prior to infection, 10 ml cultures of each mycobacterial strain were pelleted for 5 min, resuspended in PBST and sonicated twice for 10s (Laboratory Supplies, Inc., Hicksville, N.Y.). Following sonication, dispersed bacterial suspensions were allowed to stand for 5 min, and the upper 500 µl was used in subsequent infections. To ensure an infection ratio of 5 to 10 bacilli per macrophage, multiplicities of infection (MOI) were determined by adding dilutions of prepared bacilli to 1 x 10^6 differentiated THP-1 cells and allowing them to infect for 4 hours.

Colony counting. To quantify *M. tuberculosis* CFU, THP-1 cells were plated and infected as described above. At days 1, 2, 3, 5 and 8 infected cells were lysed in 1% saponin, washed with PBS, and then plated on Middlebrook 7H11 agar plates with 0.05% Tween 80, 10% OADC (GIBCO), 0.5% glycerol. Colonies were counted after 3-4 weeks of growth at 37°C.

Apoptosis assays. Cells were infected with mycobacteria at a ratio of 10 or 5 bacilli to1 macrophage for 3, 5 and 7 days. At various time points, cells were harvested in 1x PBS-0.5mM EDTA and washed twice in PBS. The cells were then fixed in 4% paraformaldehyde overnight to kill *M. tuberculosis*. Cells were labeled for TUNEL assay using the manufacturer's protocol (Boehringer Mannheim) followed by FACS analysis. Briefly, the presence of apoptosis in infected and uninfected THP-1 cells was monitored by the presence of DNA fragmentation. The *in situ* cell death detection assay detected cell death (apoptosis) by labeling DNA strand breaks in individual cells. The method uses terminal deoxynucleotidyltransferase to label free 3'OH ends in DNA with fluorescein-dUTP (TUNEL). This enzymatic labeling allows the detection of a very early apoptotic event. Apoptotic cells are then analyzed by flow cytometry.

Paraffin-embedded infected mouse tissue sections were first deparaffinized by washing the sections in two changes of xylene and then in two changes of ethanol. They were then washed sequentially in 95% ethanol, 70% ethanol, and PBS and then subjected to proteinase K digestion (25°C, 15 min). DNA strand breaks were identified by TUNEL assay using the ApopTag kit (Chemicon international) following the manufacturer's protocol using peroxidase and diaminobenzidene substrate. Hydrogen peroxide was used to quench endogenous peroxidase activity, and the cells were counterstained with hematoxylin and eosin (H&E).

Mouse infections. 7H9 broth (containing hygromycin for the knockout strains or hygromycin plus kanamycin for the complemented strain) were inoculated with frozen stocks of each strain and grown to an OD_{600} of ~1.0. The bacteria were pelleted by centrifugation, washed three times in phosphate-buffered saline containing 0.05% Tween 80, and sonicated briefly using a cup-horn sonicator. Female C57BL/6 mice (6 to 8 weeks old; Jackson Laboratories) were infected with 10^6 cfu/ml *via* tail vein injection or ~100 to 200 CFUs by the aerosol route using a nose-only aerosolization system as described earlier (Schwebach *et al.*, 2002). At various time-points after infection, mice were sacrificed and the lungs, spleens, and livers were homogenized in phosphate-buffered saline containing 0.05% Tween 80. Tissue bacterial load was assessed by plating serial dilutions of the homogenates onto 7H10 agar, containing hygromycin as appropriate for the knockout strain. Portions of the lungs, liver, and spleen were also fixed overnight in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic evaluation and photography. Four to five mice per group were sacrificed at each time point.

Results

Inhibition of macrophage apoptosis by *Rv3238c/nlaA*. The wild-type *M. tuberculosis* H37Rv and the Δ*nlaA* strain did not differ in terms of their growth kinetics in 7H9 medium. The human promonocytic THP-1 cell line is a valid *in vitro* model for studying mycobacteria induced apoptosis (Riendeau and Kornfeld, 2003). PMA differentiated THP-1 cells were infected with *M. tuberculosis* H37Rv, *M. tuberculosis* Δ*nlaA*, or the complemented strain. TUNEL labeling using *in situ* cell death detection kit (Boerhinger) followed by flow cytometric analysis were used to measure apoptosis in infected cells. Consistent with the earlier published studies, we were able to show that the avirulent strains viz. *M. bovis* BCG, *M. kansasii* were able to induce more apoptosis than the virulent H37Rv (FIG. 1). The *M. tuberculosis* Δ*nlaA* mutant induced more apoptosis than wild-type H37Rv at all the time points studied which was not a result of limited replication *in vitro* (FIG. 2A and B). We chose day 3 as the time point for all future experiments since the difference in the levels of apoptosis was most prominent at this time point. While 6.26% of cells infected with wild-type H37Rv underwent apoptosis at three days post-infection, 20.18% of cells

infected with the *M. tuberculosis* Δ*nlaA* mutant exhibited apoptosis (FIG. 3). To directly prove that this phenotype resulted from the loss of the *nlaA* gene, the Δ*nlaA* mutant was complemented with the wild-type copy of the gene and this resulted in the restoration of the apoptosis to wild-type levels ($P<0.02$) (FIG. 4). It has been shown that inhibition of apoptosis is a virulence-associated trait and attenuated strains like the H37Ra induce more apoptosis than the virulent H37Rv (Keane *et al.*, 1997). To further confirm that the phenotype was a result of the disruption of *nlaA* gene, and not a characteristic of all attenuated strains we tested the Δ*panCD* mutant, known to be attenuated in mice (Sambandamurthy *et al.*, 2002) in inducing apoptosis. Our results show that Δ*panCD* mutant induced as much apoptosis as the wild-type H37Rv strain (FIG. 4). We thus named the gene *nlaA* for Nurim Like anti-apoptotic (*nlaA*) gene based on its ability to inhibit apoptosis. It is unlikely that the differences observed were due to impaired infectivity, since the number of viable bacteria recovered per well after 4h of infection was the same for all strains tested.

Growth characteristics of Δ*nlaA* mutant in mice. We studied the ability of the *M. tuberculosis* strains to survive in the tissues of immunocompetent C57BL/6 mice by CFU analysis after intravenous and aerosol infection. The C57BL/6 strain of mice is known to restrict the proliferation of *M. tuberculosis* in tissues and would thus amplify the survival defects in attenuated strains (Orme, 2003). We used a low-dose murine aerosol infection model of chronic persistent infection (Scanga *et al.*, 2001) to examine the *in vivo* phenotype of the *M. tuberculosis* Δ*nlaA* mutant. Following an aerosol exposure of 100 bacilli in the lungs as seen by bacterial counts 24 hours post-infection, the mice were monitored for a period of 21 weeks. At 3 weeks post-infection, enumeration of the bacterial burden within the lung and spleen revealed that the Δ*nlaA* strain proliferated less than that of the wild-type H37Rv (FIG. 5). The wild-type and the mutant grew exponentially for 3 weeks post-infection and reached constant titers by 21 weeks. Strikingly, the mutant showed a growth defect and persisted at about 1 log less in the lungs in comparison to H37Rv at all time points both in the spleen and the lungs of infected mice (FIG. 5).

After intravenous challenge each mouse received about 10^4 bacteria in the lungs and about 10^5 bacteria in the spleen as seen at 24 hours post-infection. The Δ*nlaA* mutant and H37Rv strains grew exponentially for the first three weeks following infection. However, the mutant was seen to grow less than the wild-type by week 8 in the lungs of these mice. At week 34, the mutant persisted at about 10^5 bacteria in the lungs while the wild type reached a constant titer of 10^6 bacteria (FIG. 6A). A similar growth pattern was seen in the spleens of these mice, where both the mutant and wild-type grew at comparable rates for 8 weeks post-infection followed by a sharp decline in the Δ*nlaA* titers. By week 34 the Δ*nlaA* mutant reached a bacterial count of about 10^5 while the wild type persisted at constant titers (FIG. 6B). Overall, the intravenous and aerosol

infection of C57BL/6 mice shows that the deletion of *nlaA* gene in *M. tuberculosis* results in observable defects in growth and persistence demonstrating a significant attenuation of this strain.

Virulence of *M. tuberculosis* Δ*nlaA* mutant in immunocompetent mice. Another relevant measure of virulence is to assess the relative survival periods of infected mice (North, 1995). We performed time to death analysis to determine if the lesser growth of the Δ*nlaA* mutant manifested in prolonged survival of infected mice. When the C57Bl/6 mice were infected with various mycobacterial strains either by intravenous or aerosol route, mice infected with the Δ*nlaA* mutant displayed a pronounced attenuation in the time to death in both models of infection. The median time-to-death for the mutant infected via the aerosol route exceeded 225 days and that of the wild-type and the complimented strain was about 150 and 160 days respectively (FIG. 7). In mice infected intravenously, the median-time to death for the mutant was about 300 days and that of the wild-type was about 250 days (FIG. 8). The mice infected with the complemented strains intravenously survived longer than the mutant, which can be attributed to the low inoculum of bacteria in the infection as seen in growth curves of these strains in FIGS. 5 and 6. Interestingly, SCID mice infected with the mutant or the WT did not show any significant differences in mortality (FIG. 9). This result suggests that the *nlaA* gene induces protective response through the adaptive immune mechanism.

Apoptosis analysis in the mouse tissue sections. TUNEL assessment of DNA fragmentation in the spleen of mice infected with Δ*nlaA* mutant showed that about 20% of the cells were apoptotic at 3 weeks post infection (FIG. 9). In the lungs of these mice apoptosis increased markedly from 10% at 1 week to about 20% at 16 weeks post infection. In contrast, although apoptosis was identified mice infected with virulent *M. tuberculosis*, it appeared to be less than that induced by the mutant strain. By 16 weeks, mice infected with virulent bacilli exhibited minimal apoptosis (< 10%). Histopathology analysis of TUNEL stained sections of these mice are depicted in FIGS. 10 and 11. These results correlate with the reduced growth of the mutant strains as opposed to the wild type strains in the organs of intravenously infected mice (FIG. 6).

Histopathological analysis of mouse tissue sections. Granuloma formation is the key component of adaptive immune response to mycobacterial infection and is influenced by the tissue burden and the virulence of the infecting organism. At 3 weeks post-infection, histopathological analysis of lung tissue from aerosol infected mice, showed that both wild-type and mutant strain produced comparable degrees of interstitial inflammation with small scattered foci of organizing lesions (FIG. 12). At 8 and 12 weeks, granulomatous lesions began to form in both mutant and wild-type infected tissues. However, lungs of wild-type infected mice showed increasing granuloma size, coalescence of the lesions due to excessive infiltration of lymphocytes

and foamy macrophages become evident in the granuloma. The lungs of the Δ*nlaA* mutant infected mice showed retarded disease progression and showed lesser pathological evidence than that of the wild-type (FIGS. 13, 14). Analysis of the lung tissue of mice infected with wild-type at later time points of 21 weeks and 33 showed progressive granulomatous inflammation with loss of functional alveoli attributable to enlarging and coalescing lesions. The tissues from Δ*nlaA* infected mice showed smaller, less abundant and more discrete granulomas. At the level of gross pathology, there were apparent differences between tissues infected with the wild-type and mutant strain, the organs of mutant infected mice has smaller and fewer granulomas and less inflamed than that of the tissues from wild-type infected mice (FIG. 15, 16, 17).

Discussion

Based on a *phoA* reporter technology developed in our lab (Braunstein *et al.*, 2000), we have identified a novel protein of *M. tuberculosis*, NlaA. In this study, we report that a mutant of this gene can induce apoptosis of infected cells. *M. tuberculosis* infection results in intracellular survival and proliferation within the macrophages they infect (Bermudez and Goodman, 1996). Apoptosis is the cellular response to this deregulation of growth control by *M. tuberculosis* and it results in suicide elimination of mycobacteria infected cells (Molloy *et al.*, 1994). In the current study, we have consistently shown that the Δ*nlaA* mutant induces significantly more apoptosis in the THP-1 monocytic cells than its parental wild-type strain or the complemented strain. We chose to use the THP-1 cells as a model for studying apoptosis since differentiated THP-1 cells closely model the behavior of primary human alveolar macrophages (Riendeau and Kornfeld, 2003), which constitute the critical growth niche for *M. tuberculosis* after aerosol infection (Leemans *et al.*, 2001). Despite the comparable growth rates of the Δ*nlaA* mutant and the wild type in these cells, Δ*nlaA* showed more apoptosis suggesting that differential levels of cell death were independent of replication *in vitro*. This is consistent with observations by other groups that bacillary control of host cell apoptosis is a virulence-associated phenotype of *M. tuberculosis*. Keane *et al* have shown that infection of alveolar macrophages with virulent *M. tuberculosis* results in reduced levels of apoptosis and cytotoxicity as opposed to attenuated or virulent isogenic strains (Keane *et al.*, 2000). Similarly, macrophages from mice resistant to mycobacterial infection are more susceptible to *M. tuberculosis* induced apoptosis (Rojas *et al.*, 1997). Apoptosis is considered a defense strategy to limit the growth of intracellular pathogens (Moore and Matlashewski, 1994; Nash *et al.*, 1998; Vaux and Strasser, 1996). The importance of this innate defense mechanism is demonstrated in the evolutionary acquisition of apoptosis inhibition genes by many viruses (Teodoro and Branton, 1997). There is precedence among other intracellular pathogens for blocking macrophage apoptosis as a means to enable continued intracellular parasitism (Fan *et al.*, 1998; Gao and Abu Kwaik, 2000; Moore and Matlashewski,

1994; Nash *et al.*, 1998). However, for induction of apoptosis, mycobacterial effector molecule/s directly or indirectly interfere with the apoptotic pathway. The mannosylated lipoarabinomannan of *M. tuberculosis* can inhibit apoptosis (Rojas *et al.*, 2000) and the recent evidence that superoxide dismutase (SOD) diminished strains are less virulent in mice (Edwards *et al.*, 2001) suggests that apoptosis plays a role in the attenuation of mycobacteria. On the contrary, laboratories have reported that the purified protein derivative (PPD) and the 19kDa protein from *M. tuberculosis* (Lopez *et al.*, 2003; Rojas *et al.*, 1997; Rojas *et al.*, 1999) can induce apoptosis of human monocytes. Therefore, regulation of mechanism of phagocytic cell death by *M. tuberculosis* is a multifactorial process.

In an attempt to further characterize the *nlaA* gene, the growth of the mutant strain was evaluated in C57/BL6 mice model. Wild-type *M. tuberculosis* H37Rv and the Δ*nlaA* mutant replicated equally well in the 7H9 broth. The Δ*nlaA* however showed reduced growth during the acute phase of infection in the lungs and spleen of mice infected via the aerosol route. The mutant was maintained at titers less than that of the wild-type till 21 weeks after which the experiment was terminated. In mice infected intravenously the mutant and the wild type grew to similar titers for the first three weeks post infection followed by a decline in the mutant titers. The Δ*nlaA* mutant was maintained at titers less than that of the wild-type in both the spleen and the lungs of infected mice up to 34 weeks. Whereas, the Δ*nlaA* mutant strain grew at rates similar to that of the *nlaA* proficient strains in THP-1 cells and bone marrow derived macrophages from C57BL/6 mice (data not shown), in animals the mutant showed a decline in growth in organs of infected mice. This suggests that the reduced growth of the Δ*nlaA in vivo* did not result primarily from a lower intrinsic capacity to replicate, but due to the interaction between mycobacteria and the host. Using the same model of immunocompetent mice we were able to demonstrate the prolonged survival, over 50 days, of mice infected with Δ*nlaA* intravenously or via aerosol route.

A complex balance exists: *M. tuberculosis* can inhibit apoptosis in directly infected cells to facilitate intracellular replication or survival and alternatively, the host needs to induce apoptosis in these cells to inhibit microbial replication. *M. tuberculosis* disturbs this balance, as evidenced in patients infected with the tubercle bacilli. Monocytes from patients with TB undergo apoptosis, as well as necrosis after infection with virulent *M. tuberculosis*, whereas monocytes from PPD+ healthy donors undergo only apoptosis, which was more than the level of apoptosis in infected patients (Gil *et al.*, 2004). In one of the first descriptions of apoptosis, cells undergoing this form of cell death showed reduction in the viability of BCG, whereas necrotic death of these cells resulted in no intracellular mycobacterial inhibition (Molloy *et al.*, 1994). Classical descriptions of histology of the tuberculous granuloma refer to caseous necrosis at tissue level and "burst" of bacilli laden macrophages as the cause of bacterial dissemination and tissue

damage (Dannenberg, 1993). Since apoptotic cells can kill the tubercle bacilli *in vitro* (Duan *et al.*, 2001; Molloy *et al.*, 1994; Oddo *et al.*, 1998), and owing to the marked differences in levels of apoptosis *in vitro* that was unique to infection with the Δ*nlaA* mutant, we assumed that apoptosis is the factor for the reduced growth rate of the mutant *in vivo*. Apoptosis was prominent among the cells in the spleen and lungs of mice infected with the Δ*nlaA* strain but reduced in mice infected with virulent *M. tuberculosis* H37Rv. This increased rate of apoptosis correlated with the fall in bacillary load seen in the spleen and the lungs over time, compared to the rapid rise by virulent *M. tuberculosis* strains. Further, our histopathology analysis on the hematoxylin and eosin staining stained sections of infected mice, shows pathological disease in the tissues of mice infected with the wild-type *M. tuberculosis*. Overt changes were seen in the lungs of mice infected with the Δ*nlaA* mutant as compared to wild type, where the granulomas were clearly visible. In the lungs of *M. tuberculosis* infected mice the granulomas were discretely spread over the lung area and seemed to coalesce in later stages of infection. The mutant infected lungs had smaller and less abundant granulomas as were the other tissues infected with this mutant. Since the Δ*nlaA* mutant exhibited suppressed bacterial replication and pathological disease in the organs of mice infected, at the same time being less pathogenic to these mice as reflected by their extended survival periods, it is feasible to say that the mutant induced apoptosis contributes to the attenuation of virulence through restricting the bacteria in the mouse lungs. It can be reasoned that in the absence of apoptosis, the bacilli continue to replicate, and the host cell eventually explodes as a result of membrane damage.

Host induction of apoptosis have other advantages in addition to controlling intracellular replication, as apoptotic macrophages can be used as a source of antigen for presentation by bystander dendritic cells (Schaible *et al.*, 2003; Yrlid and Wick, 2000). Therefore, in addition to impairing the innate immune response by inhibition of apoptosis and intracellular survival, *M. tuberculosis* affects the adaptive response by this host evasion strategy. This is indeed possible since SCID mice infected with the wild type and the Δ*nlaA* mutant displayed identical mortality rates, which explains the limited growth rate Δ*nlaA* during the adaptive phase of the immune response. Therefore, protective immunity in these mice infected with the *nlaA* mutant is a result of innate and acquired defense mechanisms.

Immunity induced by the Δ*nlaA* mutant was compared with immunity induced by a wild type *M. tuberculosis*. The Δ*nlaA* mutant strain induced greater immunity, as determined by CD4+ T cells reactive against two separate antigens. See FIGS. 18 and 19.

Example 2. *Mycobacterium tuberculosis nuoG* is a virulence gene that inhibits apoptosis of infected host cells

Mycobacterium tuberculosis is an extremely successful pathogen that has already infected approximately one third of the world's population and is currently estimated to cause 8 million new infections and 2-3 million deaths annually (Dye et al., 1999). The survival and persistence of *M. tuberculosis* depends on its capacity to manipulate multiple host defense pathways (Nguyen and Pieters, 2005). The genetic predisposition of the host in defense against mycobacterial infections is linked to the capacity of the macrophage to undergo apoptosis or necrosis upon infection, with the former response imparting a resistant and the latter a susceptible host phenotype (Pan et al., 2005). To determine the role of the inhibition of macrophage apoptosis for virulence of *M. tuberculosis*, we identified *M. tuberculosis* genetic loci necessary and sufficient for the inhibition of macrophage apoptosis. Here we show that the *nuoG* gene, which encodes a subunit of the NADH-Dehydrogenase Complex I of *M. tuberculosis*, is necessary for the inhibition of infection-induced apoptosis of macrophages and for virulence of the bacteria in the mouse model of tuberculosis. In addition, our results demonstrate that apoptosis is an important part of the host innate immune response to mycobacterial infections.

Virulent species of mycobacteria have established mechanisms to counter the host cells effort to undergo apoptosis by inhibiting infection-induced apoptosis, a capacity that is not found in non-virulent species, and therefore a correlation between virulence and inhibition of macrophage apoptosis was proposed (Bakcewicz –Sablinska et al., 1998; Keane et al., 2000; Oddo et al., 1998; Sly et al., 2003). In the present study a "gain-of-function" genetic screen was established using the non-pathogenic M. smegmatis mc2155 because it is a fast-growing Mycobacterium that has been shown to be highly transformable (Snapper et al., 1990) and safe for use in studies of *M. tuberculosis* gene expression using standard BSL-2 laboratory practices (Bange et al., 1999). In our initial studies, we demonstrated that *M. smegmatis* mc2155 induced a very strong and rapid apoptosis response of a differentiated human macrophage-like cell line (PMA treated THP-1 cells) when compared to *M. bovis* BCG infected or uninfected macrophages (FIG. 20A). This result established the potential of *M. smegmatis* mc2155 to be used in the "gain-of-function" screen, and subsequently bacteria were transformed with a cosmid library of *M. tuberculosis* genomic DNA. A total of 312 individual random clones of *M. smegmatis* transformed by *M. tuberculosis* cosmids were screened for their ability to inhibit infection-induced apoptosis of THP-1 cells when compared to wild type *M. smegmatis* (FIG. 23). Assuming random representation of *M. tuberculosis* sequences by the cosmid transformants, 312 cosmid clones suggests that over 95% of the *M. tuberculosis* genes were represented. After three successive screens using bright field microscopy or flow cytometry to assess levels of cell death in infected THP-1 cells, 12 clones with markedly reduced cell death promoting activity were selected (FIGS. 20A and 23). The capacity of these clones to inhibit apoptosis was analyzed

using a specific assay for apoptosis (TUNEL assay) followed by flow cytometry to determine the percentage of apoptotic cells (FIGS. 20A and B). Finally, three clones (designated K20, J21 and M24) showing the strongest inhibition of *M. smegmatis*-induced apoptosis in macrophages were selected.

The inserts of the three cosmids were analyzed by DNA sequencing and restriction enzyme digestion and found to be non-overlapping (FIGS. 23 and 24).

It was theoretically possible that genomic mutants of *M. smegmatis* that had lost their ability to induce apoptosis were selected during the screening process. To address this possibility, the episomal cosmid DNA of the three selected clones was extracted and again transfected into *M. smegmatis* mc2155. The capacity of the corresponding clones together with the original clones to induce apoptosis was analyzed. In all three cases the re-transformed clones had the same phenotype as the original clones, all leading to about 50% reduction of apoptosis when compared to wild-type bacteria (FIG. 20C). Thus, the phenotype of reduced apoptosis-induction was due to the transfected cosmid DNA and not to random genomic mutations in the *M. smegmatis* genome. Nevertheless, overall the effect of the cosmids on macrophage apoptosis was modest (about 50% reduction) which may have been due to the very strong capacity of *M. smegmatis* to induce apoptosis when compared to other mycobacteria (see FIG. 20A). We therefore tested the effects of the *M. tuberculosis* cosmids on apoptosis induction by another mycobacterial species, *M. kansasii*. This opportunistic pathogen is known to be a strong apoptosis-inducing mycobacterium, but it shows slower host cell killing than *M. smegmatis* with little apoptosis after one to three days of infection and a significant rise after 5-7 days of infection (data not shown and Balcewicz-Sablinska et al., 1998). Therefore we transformed the three isolated cosmid clones into *M. kansasii* to generate *M. kansasii*-J21, -M24, -K20, and an empty cosmid vector control to generate the *M. kansasii* control strain (*M. kansasii*-CO). THP-1 cells were harvested after 5 days of infection and the apoptosis assay by TUNEL staining and flow cytometry was performed. *M. kansasii* wild-type induced 95% apoptosis while the *M. kansasii*-J21, -K20 and -M24 showed 16%, 20% and 17% apoptosis respectively compared to 86% of apoptosis induced by *M. kansasii*-CO which was a significant reduction ($p<0.01$, unpaired t-test) (FIG. 20D). *M. tuberculosis* H37Rv was used as a positive control that induced apoptosis only in 11% of the cells which was very similar to the 6% of apoptosis observed in untreated cells. Interestingly, all three cosmids transfected into *M. kansasii* reduced the apoptosis induction to a level very similar to *M. tuberculosis*.

Apoptosis of infected macrophages has been reported to be a pathway to either directly kill ingested bacteria (Molloy et al., 1994) or to facilitate killing of bacteria within apoptotic bodies phagocytosed by bystander macrophages (Fratazzi et al., 1997). Therefore we

hypothesized that inhibition of apoptosis is important for mycobacteria in order to evade the host's innate immune response. The very strong inhibition of apoptosis observed for *M. kansasii* clones transformed with *M. tuberculosis* cosmids allowed investigation of this hypothesis.

SCID mice (BALB/c background) were infected with the *M. tuberculosis* cosmid transformed *M. kansasii* clones (*M. kansasii* -J21, -K20, and -M24) intravenously. In addition mice were infected with *M. tuberculosis* H37Rv and the *M. kansasii*-CO as positive and negative controls. The median survival of SCID mice infected with *M. tuberculosis* H37Rv was 15 days, whereas median survival for mice infected with *M. kansasii*-CO was >200 days, since only 2 out of 7 mice died by the end of the observation period of 200 days (FIG. 21A). The mice infected with *M. kansasii*-J21 -M24 had a median survival of 44 days or 60 days, respectively. The survival curves for both of these bacteria were very significantly different from the survival curve of *M. kansasii*-CO infected mice (p=0.0002, logrank test). Surprisingly, most of the *M. kansasii*-K20 infected mice survived the length of the experiment (2 out of 7 died), similar to *M. kansasii*-CO infected mice (p = 0.87).

The results of the survival study were consistent with the data on the bacterial growth of the different strains in lung, liver and spleen of infected mice as analyzed after 24 hours, 2 and 5 weeks. All bacteria initially infected the analyzed organs in very similar numbers as determined by colony forming units (cfu) at the 24h harvest (FIG. 21B). After two weeks, differences became apparent, most notably in the lung and spleen where mice infected with *M. tuberculosis* H37Rv showed a 10-fold increase in bacterial load, compared to *M. kansasii*-J21 and –M24 which remained the same and *M. kansasii*-K20 and *M. kansasii*-CO which showed a 10-fold decrease in cfu. After five weeks all *M. tuberculosis* infected mice were dead, the *M. kansasii*-J21 and –M24 infected mice had a 10-fold increase in cfu in the lungs and spleens, and the *M. kansasii*-CO and –K20 infected mice had a drastic reduction of bacterial growth in all three organs with about 100-1000 fold less cfu compared to the initial colonization (FIG. 21B), and this reduction continued until no bacteria could be detected after 10 weeks of infection.

Analysis of the histopathology of the lungs revealed that after 14 days the lung morphology was well preserved in all groups of mice (FIG. 21C), but after 35 days the lungs of *M. kansasii*-J21 infected mice were almost completely consolidated and in *M. kansasii*-M24 infected mice the lungs showed significant infiltration of cells. In contrast, lungs of mice infected with *M. kansasii*-K20 and *M. kansasii*-CO showed no signs of infiltration and had normal morphology even after 35 days. Interestingly, the lung pathology of *M. kansasii*-J21 correlated with early killing of mice when compared to the less severe pathology in *M. kansasii*-M24 infected mice, which also died later than *M. kansasii*-J21 infected mice. In order to correlate the virulence of the different strains with their capacity to inhibit apoptosis, lung sections obtained 14

days after infection were stained for apoptotic cells using a TUNEL based assay. Clearly, *M. tuberculosis*, *M. kansasii*-J21 and- *M. tuberculosis*-M24 did not induce a strong apoptotic response in contrast to *M. kansasii*-K20 and –CO infected lungs (FIG. 21D). Quantification of apoptotic cells demonstrated a very low level of apoptotic cells in *M. tuberculosis* infected lungs (1-2%), a slight increase in *M. kansasii*-J21 and *M. tuberculosis*-M24 infected lungs (~5%) and a strong increase in *M. kansasii*-K20 (17%) and -CO (26%) infected lungs. Our results indicated that *M. tuberculosis* cosmids J21 and M24 mediated inhibition of host cell apoptosis in human THP-1 cells and also *in vivo* in the lungs of SCID mice. In contrast, cosmid K20 was similarly active in THP-1 cells but failed to show a significant effect in the *in vivo* mouse studies.

Since the cosmid J21 mediated the strongest increase in virulence of transfected M. *kansasii*, it was selected for further analysis. The sequence of the *M. tuberculosis* DNA was determined to start at bp 3511794 and finish at bp 3545572 as annotated in Cole et al., 1998 (FIG. 24). A literature search for *M. tuberculosis* genes implicated in virulence revealed that several genes encoded in this portion of the genome, including *PPE53*, *Rv3163c*, *Rv3173c* and *Rv3174*, are upregulated by *M. tuberculosis* within macrophage phagosomes (Schnappinger et al., 2003). Another gene included in this interval, *Rv3168*, has been identified by transposon mutagenesis as important for survival of *M. tuberculosis* in infected mice (Sassetti and Rubin, 2003). We thus generated deletion mutants of *M. tuberculosis* H37Rv of each of these genes by specialized transduction (Bardarov et al., 2002)(FIG. 24). Furthermore, the *nuo* operon which is encoded by the first 15 kbp of the cosmid insert was selected to be disrupted by deletion of *nuoG* in *M. tuberculosis*. In addition, a mutant deleted in the region containing the genes *PPE53* to *Rv3163c* was created (FIG. 24).

The impact of the deletion from M. tuberculosis of specific genes contained in cosmid J21 was analyzed initially using SCID mice. This showed that deletion of *nuoG* had the strongest effect on *M. tuberculosis* virulence, since median survival was increased from 16 days for wild-type *M. tuberculosis* to 31days for the *nuoG* mutant ($p = 0.0031$ as determined by the logrank test). All of the other deletions had minimal effects with the median survival of the mice ranging from 17-18 days, except for Δ*Rv3174* strain, which showed a median survival of 21 days. This was significantly different from *M. tuberculosis* ($p = 0.0031$), and although a subtle effect, might also be of biological importance. Selected deletion clones were also analyzed for their capacity to inhibit infection-induced apoptosis using differentiated THP-1 cells. The deletion of *nuoG* resulted in a strong increase in apoptosis induction from 5% with wild-type bacteria to 25% with the *nuoG* bacteria ($p=0.0027$, two-tailed t-test). All of the other deletion clones showed only a slight increase of apoptosis induction (8-12%) that was statistically significant ($p<0.05$, two-tailed t-test) when compared to *M. tuberculosis* but was most likely of no biological importance since

for example the Δ*PPE53/Rv3163c* deletion induced the most apoptosis second to Δ*nuoG* but had no effect on the virulence of the bacteria as documented by the survival experiment in FIG. 22A. Altogether, the results suggested that *nuoG* was a virulence gene that mediated most or all of the anti-apoptotic properties of the cosmid clone J21. To further confirm this conclusion, the *nuoG* strain was complemented with an integrating plasmid carrying a copy of *nuoG* behind a constitutively active promoter, and compared to wild-type and Δ*nuoG* strains for their capacity to kill infected SCID mice. The median survival times for mice infected with *M. tuberculosis* wild-type or the complemented strain were not significantly different (14 d and 16.5 d, respectively). In contrast, mice infected with Δ*nuoG* bacteria survived twice as long as those infected with wild-type bacteria (median survival of 27.5d, $p<0.0001$ using the logrank test (FIG. 22C)).

In conclusion, the "gain-of-function" genetic screen successfully led to the identification of the first *M. tuberculosis* gene to our knowledge to be implicated in the inhibition of infection-induced macrophage apoptosis. The gene, *nuoG*, is part of the NADH-dehydrogenase complex I that consists of 14 proteins encoded by one operon. A *nuoG* mutant of *Salmonella* serotype Gallinarum has previously been shown to protect chickens against experimental fowl typhoid (Zhang-Barver et al., 1998). The nuoG subunit is most likely localized on the extracellular part of the protein complex (Friedrich and Bottcher, 2004). Currently, it is not clear which if any specific genes of the operon are needed in addition to *nuoG* for the function of apoptosis inhibition. While the mechanism by which *nuoG* may directly or indirectly lead to inhibition of host cell apoptosis remains to be determined, it is intriguing that other studies have implicated host cell mitochondria as a target for bacterial virulence and host response evasion strategies (Blanke, 2005). In this regard, it is interesting that the mammalian ortholog of *nuoG* is a substrate of caspase-3 cleavage, which mediates initiation of pro-apoptotic events culminating in the loss of mitochondrial potential (Ricci et al., 2004). This suggests the possibility that the product of the *nuoG* gene of *M. tuberculosis* might be capable of replacing the function of its mammalian counterpart, thus maintaining the mitochondrial electron transport chain and blocking the cascade of events leading to apoptosis.

The capacity of M. tuberculosis to inhibit host cell apoptosis was suggested by earlier studies to be a virulence factor (Bakcewicz –Sablinska et al., 1998; Keane et al., 2000; Oddo et al., 1998; Sly et al., 2003), a hypothesis that is strongly supported by the more recent demonstration of the importance of host cell apoptosis induction for resistance or susceptibility to tuberculosis (Pan et al., 2005). Our results demonstrate for the first time, through the use of well-defined bacterial mutants, the importance of apoptosis inhibition for the virulence of *M. tuberculosis* and the major role that host cell apoptosis has in the innate immune response to *M. tuberculosis* infections.

Materials and Methods

Bacteria and culture conditions. *M. smegmatis* (mc2155) (Snapper et al., 1990), *M. kansasii* (ATCC 12478) and *M. tuberculosis* strain H37Rv (ATCC 25618). GFP expressing BCG and *M. smegmatis* were generated by subcloning the enhanced GFP gene (BD Bioscience Clontech) into the mycobacterial expression vector pMV261. The new plasmid named pYU921 was transfected into competent cells by electroporation as in Snapper et al., 1990. All bacteria were cultured as described in Snapper et al., 1990 and Bange et al., 1999

Generation of a genomic library of M. tuberculosis in the cosmid vector pYUB415. The strategy has been previously described in Bange et al., 1999. Briefly, M. tuberculosis (strain Erdman) genomic DNA was purified and partially digested with *Sau*3A. DNA fragments of about 40 kbp were selected by agarose gel purification and ligated into arms of cosmid vector pYUB415 digested with *Xba*I and *Bam*H1 prepared as in Bange et al., 1999. DNA was packaged in vitro with Gigapack XL (Stratagene) and *E. coli* were transduced and selected on LB-plates containing 100 μg/ml ampicillin. Over 105 independent clones were pooled, and DNA for transformation was obtained using standard alkaline lysis method.

Tissue Culture conditions and Infection. Human myelomonocytic cell line THP1 (ATCC TIB-202) was cultured and differentiated using phorbol myristate acetate (PMA)(Sigma) as in Dao et al., 1991. Bacteria were grown to an OD_{600} ranging from 0.5 to 0.8 and were sonicated twice for 20s using a cup horn sonicator (Laboratory Supplies, Inc.) and allowed to settle for 10 min. The infection was carried out at a multiplicity of infection (MOI) of 10:1 (10 bacilli to 1 cell) for 4 hours in triplicate wells. After 4 hours, the extracellular bacteria were removed by four washes with phosphate buffered saline (PBS). The cells were incubated in DMEM (Invitrogen) with 20% human serum (Sigma) and 100 μg/ml of gentamycin (Invitrogen) and apoptosis assay was performed.

Apoptosis Assay. A TUNEL assay was performed to reveal apoptosis-induced DNA fragmentation using the In Situ Cell Death Detection Kit-Flourescein (Roche Applied Sciences). Triplicate wells were either pooled after infection (FIGS. 20B and C) or analyzed independently (FIG. 20D and 22B). The assay was carried out as described in Dau et al., 1991 and the percentage of stained cells was analyzed using flow cytometry.

Transformation and gain-of function screen. Transformations were performed by electroporation of competent mycobacteria as described in Snapper et al., 1990. For the initial screen, *M. smegmatis* was transformed with the genomic DNA cosmid library described above and 312 cosmid clones were picked and grown in liquid medium containing 50 μg/ml hygromycin. Differentiated THP-1 cells were infected and the effect of the cosmid clones on the

monolayer of cells was compared to that of wild-type *M. smegmatis* by microscopy and subsequently by FACS analysis of PI or TUNEL stained cells (FIG. 23).

Four successive rounds of screening identified 4 clones of greatest interest, and their cosmid DNA was purified and screened by restriction digest (FIG. 23). For cosmid J21, the 5' and 3' ends of the insert-DNA were sequenced and aligned with the published genomic *M. tuberculosis* DNA sequence (FIG. 24).

Specialized transduction and complementation. Specific genes of *M. tuberculosis* were disrupted using specialized transduction as described in Bardarov et al., 2002. For the *nuoG::hyg*-null allele, the hygromycin resistance cassette was introduced between the first 4 bp of the nuoG 5'end and the last 163 bp of the 3'end of the open reading frame. The successful deletion of the gene was demonstrated by Southern blotting as described previously (FIG. 25). For complementation of the Δ*nuoG* strain, the open reading frame of *nuoG* was amplified by PCR and subcloned behind a constitutive promoter into the plasmid pMV361, which allows integration into the genome of *M. tuberculosis* (Stover et al., 1991).

Animal studies. BALB/c or SCID/Ncr (BALB/c background) mice (4-6 weeks old females, purchased from the NCI) were infected intravenously through the lateral tail vein with $1x10^6$ bacteria. For survival studies groups of 10 mice were infected, and after 24 h three mice per group were sacrificed to determine the bacterial load in the organs. In order to follow the bacterial growth an additional three mice per timepoint were infected. The organs (lung, spleen, liver) were homogenized separately in PBS/0.05% TWEEN-80, and colonies were enumerated on 7H10 plates grown at 37°C for 3-4 weeks. For histopathology, tissues were fixed in 10% buffered formalin and embedded in paraffin; 4 μm sections were stained with haematoxylin and eosin. TUNEL staining was performed on the paraffin-embedded tissue sections using the In Situ Cell Death detection kit, POD (Roche Applied Sciences) as per the manufacturer's protocol. Quantification was performed blinded by counting the amount of apoptotic cells per ~200 total cells in 8 separate areas of two lung sections for each of the three mice per group. All animals were maintained in accordance with protocols approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

Statistical Analysis. All statistical analysis was performed using PRISM® 4 (GraphPad Software).

Example 3. Immunological evaluation of Δ*nlaA* and Δ*secA2* mutants.

The ability of ΔnlaA (Example 1) and ΔsecA2 mutants (Braunstein et al., 2003) to induce host apoptosis and immunity to mycobacteria was evaluated using immunological and flow cytometric methods.

THP-1 cells (human myeloid/monocytic cell line) were infected with *M. tuberculosis* strains H37Rv, ΔRD1 (Hsu T, et al.,2003), ΔnlaA and ΔsecA2 at an MOI of 10:1. The cells were harvested after 60 hours, and analyzed by TUNEL as follows. The cells were stained with the In Situ Cell Death Kit (Roche), which labels strand breaks by the terminal deoxynucleotidyl transferase-mediated addition of fluorescein dUTP to free 3'-OH DNA ends. The size and complexity of the infected THP-1 cells was assessed by measurement of forward and side light scattering, and DNA DNA fragmentation was assayed by determination of fluorescein incorporation using fluorescence-activated cell sorter (FACS) (Schrijvers et al., 2004) THP-1 cells infected with ΔnlaA or ΔsecA2, but not Rv or ΔRD1, showed extensive apoptosis (FIGS. 26 and 27).

The *M. tuberculosis* SecA2 gene functions in superoxide dismutase A (SodA) secretion (Braunstein et al., 2003). Deleting *SecA2* from virulent *M. tuberculosis* attenuates the virulence of the mycobacterium in mice. To evaluate whether the SecA2 superoxide dismutase prevents apoptosis, SodA activity was restored by transfecting an *M. tuberculosis* ΔsecA2 mutant with an αSodA plasmid construct (FIG. 28A). That vector features an Hsp60 promoter and an Ag85 signal sequence driving expression of a SodA gene (Braunstein et al., 2000). This plasmid drives the constitutive expression of a SodA protein that is secreted by the SecA1 system. This provides a SodA protein that is not dependent on SecA2 and thus complements the defective secretion of SodA from the ΔSecA2 mutant. THP-1 cells were infected with the wild type *M. tuberculosis* H37Rv, the *ΔSecA2* mutant, and the *ΔSecA2*- αSodA SOD restored strain and the extent of apoptosis of the THP-1 cells was evaluated using TUNEL as described above. As shown in FIG. 28B, the ΔSecA2 mutant induced apoptosis, but the ΔSecA2- αSodA strain restored the ability of the mycobacterium to inhibit apoptosis. This indicates that the secretion of SodA protein can be directly responsible for inhibition of infection-induced apoptosis.

Apoptosis is believed to function in host defense by making pathogen antigens available for presentation by bystander dendritic cells (Schaible et al., 2003; Yrlid and Wick, 2000). To determine if macrophages present more pathogen antigens when infected with mycobacteria that induce apoptosis than when infected with mycobacteria that do not induce apoptosis, the apoptosis-inducing mycobacterial strains Δ*secA2* and Δ*nlaA*, and the apoptosis-suppressing strains H37Rv (virulent), Δ*RD1* and Δ*panCD* (attenuated virulence - See International Patent Publication No. WO 03/070164 A2, incorporated herein by reference) were transfected with plasmid constructs comprising the 19kDA lipoprotein antigen fused to the coding sequence for amino acid residues 252-269 (SIINFEKL) of chicken ovalbumin (OVA) under the control of the Hsp 60 promoter (FIG. 29), creating ΔsecA2-19k-OVA, ΔnlaA-19k-OVA, H37Rv-19k-OVA, ΔRD1-19k-OVA and ΔpanCD-19k-OVA. Each of these strains was combined with bone

marrow-derived macrophages to allow infection of the macrophages, then T cells from TCR transgenic OT-1 mice (SIINFEKL / H-2K^b specific) were added and interferon-γ (IFN γ) was measured by ELISA. The OVA peptide antigen is presented on the MHC Class I H-2K^b molecule on the macrophages, where the OVA antigen is recognized by the T cell receptor on OT-1 cells, causing production of IFNγ (FIG. 29). As shown in FIG. 30, only the strains that allow host apoptosis, ΔsecA2-19k-OVA and ΔnlaA-19k-OVA, stimulated OT-1 production of IFNγ.

The ability of the above-described mutants expressing the transgenic 19k-OVA fusion protein to stimulate splenocyte proliferation in vivo was determined by injecting OT-1 splenocytes (Thy 1.2+) labeled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) into Thy1.1 congenic mice then, after 24 hours, infecting the mice with the mutant mycobacterium. After 5-7 days, the mice were sacrificed and their splenocytes were analyzed by FACS to determine the intensity of CFSE fluorescence in the Thy1.2$^+$ T cells (FIG. 32). The CFSE enters the cells and binds irreversibly to intracellular and cell surface proteins by reaction with amines. When cells divide, the fluorescence intensity of each daughter cell is halved, so a proliferating cell population (e.g., antigen-activated T cells) exhibits a flow cytometry fluorescence profile as shown at the bottom right of FIG. 32. Results of these experiments are shown in FIG. 33. Only the strains that allow host apoptosis, ΔsecA2-19k-OVA and ΔnlaA-19k-OVA, exhibited OT-1 T cell activation *in vivo*. This indicates that apoptosis of infected cells promotes priming and activation of T cells against mycobacterial antigens.

The ability of the apoptosis-inducing mutants to induce an immune response to mycobacterial antigens was further evaluated with the *in vivo* cytotoxic T lymphocyte (CTL) assay outlined in FIG. 34. Mice were immunized with the 19k-OVA antigen mycobacterial strains described above. After 7-10 days, the mice were then injected with 10^7 Thy1.2$^+$ T cells that were either labeled with (a) a low concentration of CFSE and the OVA SIINFEKL peptide, or (b) a high concentration of CFSE without the OVA peptide. About 36 h later, the mice were sacrificed and FACS analysis was performed to quantify low and high-intensity fluorescing lymph node cells. Where the mouse has a CTL response specific for the OVA peptide, that response would kill only the CFSE^{lo} cells; the CFSE^{hi} cells should be unaffected and serve as a control.

FIG. 35 shows results of that assay. The mice immunized with the apoptosis-inducing mycobacteria mounted a CTL response to the OVA antigen-bearing T cells, whereas the mice immunized with the analogous mycobacteria that did not induce apoptosis did not mount the CTL response.

Example 4. Inoculation with *Mycobacterium tuberculosis* mutants that inhibit host apoptosis attenuates infection with virulent *Mycobacterium tuberculosis*

Mice were vaccinated with 10^6 organisms of *M. bovis* BCG, the transgenic *M. tuberculosis* with the *nlaA* deletion described in Example 1 (Δ*nlaA* in Table 1), a transgenic *M. tuberculosis* combining the Δ*secA2* and Δ*nlaA* deletions described above (Δ*secA2*/Δ*nlaA*), and a transgenic *M. tuberculosis* having deletions in *RD1* and *pan*, as described in International Patent Publication No. WO 03/070164 A2, incorporated herewith by reference, and expressing a transgenic listeriolysin (as in Grode et al., 2005)(Δ*RD1*/Δ*pan*-listeriolysin). Two months following vaccination, the mice were challenged with 200 CFUs of *M. tuberculosis* Erdman by aerosol route. TB growth in the lungs and spleens were evaluated at 1 month and 3 months post-challenge. Results are provided in Table 1.

Table 1. Values are in $\log_{10}$.

1 month:	Lungs	Spleens
Naive	6.30 ± 0.06	5.35 ± 0.07
BCG	4.92 ± 0.07(-1.38)	4.24 ± 0.18(-1.11)
Δ*nlaA*	4.52 ± 0.15(-1.78)	4.04 ± 0.24(-1.31)
Δ*secA2*/Δ*nlaA*	5.25 ± 0.08(-1.05)	4.27 ± 0.20(-1.08)
3 months:		
Naive	5.43 ± 0.02	4.93 ± 0.10
BCG	4.92 ± 0.14 (-0.51)	4.31 ± 0.11 (-0.62)
Δ*nlaA*	4.56 ± 0.16 (-0.87)	3.79 ± 0.26 (-1.14)
Δ*secA2*/Δ*nlaA*	5.17 ± 0.14 (-0.26)	4.88 ± 0.09 (-0.05)

In a second experiment using the same protocol, protection with *M. tuberculosis* Δ*secA2* and Δ*nlaA*, along with BCG, was evaluated (Table 2).

Table 2.

Group	1 mo. lung	1 mo. spleen	3 mo. lung	3 mo. spleen
naive	6.67±0.08	4.61±0.04	5.78±0.02	4.79±0.03
Δ*secA2*	4.77±0.07 (-1.90)	3.84±0.11 (-0.77)	5.16±0.05 (-0.62)	4.49±0.15
Δ*nlaA*	5.34±0.08 (-1.33)	4.04±0.19 (-0.57)	5.38±0.09 (-0.40)	4.67±0.07
BCG	5.52±0.02 (-1.15)	3.76±0.08 (-0.85)	5.73±0.14	4.67±0.09

Cytokine induction by BCG, the Δ*nlaA* mutant, and the Δ*secA2*/Δ*nlaA* mutant was also determined. Two months after vaccination with 10^6 organisms, mice were challenged with 200

CFUs of *M. tuberculosis* Erdman by aerosol route. At 10 days post-challenge, lung cells were removed and analyzed for cytokine message using real-time PCR (cells directly without *in vitro* stimulation). The results are reported in Table 3 as message levels relative to the GAPDH housekeeping gene.

Table 3.

Expt. Group	IFN-γ	IL-12	IL-4
Naive	4.0	30	0.8
BCG	27	25	0.3
Δ*nlaA*	32	16	0.3
Δ*secA2*/Δ*nlaA*	39	20	1.3

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

SEQ ID NOS

SEQ ID NO:1 - nlaA amino acid sequence

MKRYLTIIYGAASYLVFLVAFGYAIGFVGDVVVPRTVDHAIAAPIGQAVVVNLVLLGVFA
VQHSVMARQGFKRWWTRFVPPSIERSTYVLLASVALLLLYWQWRTMPAVIWDVRQPAGRV
ALWALFWLGWATVLTSTFMINHFELFGLRQVYLAWRGKPYTEIGFQAHLLYRWVRHPIML
GFVVAFWATPMMTAGHLLFAIGATGYILVALQFEERDLLAALGDQYRDYRREVSMLLPWP
HRHT

SEQ ID NO:2 - *nlaA* cDNA sequence

atgaagcgttatttgacgatcatttacggggccgcgagctatctggtattcctggttgcc
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caccggcatacctga

SEQ ID NO:3 – nuoG amino acid sequence

MVTLTIDGVEISVPKGTLVIRAAELMGIQIPRFCDHPLLEPVGACRQCLV
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LCARCTRFSDQIAGDPFIDMQERGALQQVGIYADEPFESYFSGNTVQICP
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LAALLVGGIEPADFADPDAVLAALDATGFVVSLELRHSTVTERADVVFPV
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GVPTVEAAREELAALGIWDGKHAAGPHIAATGPTQPEAGEAILTGWRMLL
DEGRLQDGEPYLAGTARTPVVRLSPDTAAEIGAADGEAVTVSTSRGSITL
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SEQ ID NO:4 – *nuoG* cDNA sequence

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CACGGTGTGATGGAACTGCTGCTGATCAACCATCCGCTGGATTGCCCGAT

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ACCGACCATCGCCGCGGCAAGGTGCTGCGGCGGCTGGCCGGTGACGACCC
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GACGACCCAGAAAGCCGGCGCGTTCGTCAACTGGGAGGGTCGCTACCGTA
CATTCGAACCCGCGCTGCGCGGCAGCACACTGCAAGCTGGCCAGTCGGAT
CACCGGGTGCTGGACGCGTTGGCCGACGACATGGGTGTCCATCTGGGCGT
GCCCACCGTGGAGGCGGCCCGCGAGGAGCTGGCCGCGCTCGGTATCTGGG
ACGGCAAACACGCTGCCGGTCCCCACATCGCGGCCACCGGGCCGACCCAA
CCCGAAGCTGGTGAGGCGATCTTGACCGGGTGGCGGATGCTCCTCGACGA
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CGGGCTCGACGGTGCACCGACAGCTGAGGGTGACAATCGGCAGCATCGTG
AAAATCGGAGCGGGCTCATGA

SEQ ID NO:5 – SecA2 amino acid sequence of *M. tuberculosis* - GenBank Accession P66785

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  1 mnvhgcpria acrctdthpr grpafayrwf vpkttraqpg rlssrfwrll gasteknrsr
 61 sladvtasae ydkeaadlsd eklrkaagll nlddlaesad ipqflaiare aaerrtglrp
121 fdvqllgalr mlagdviema tgegktlaga iaaagyalag rhvhvvtind ylarrdaewm
181 gplldamglt vgwitadstp derrtaydrd vtyasvneig fdvlrdqlvt dvndlvspnp
241 dvalideads vlvdealvpl vlagtthret prleiirlva elvgdkdade yfatdsdnrn
301 vhltehgark vekalggidl yseehvgttl tevnvalhah vllqrdvhyi vrddavhlin
361 asrgriaqlq rwpdglqaav eakegiette tgevldtitv qalinryatv cgmtgtalaa
421 geqlrqfyql gvspippnkp niredeadrv yittaakndg ivehitevhq rgqpvlvgtr
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481 dvaeseelhe rlvrrgvpav vlnakndaee arviaeagky gavtvstqma grgtdirlgg
541 sdeadhdrva elgglhvvgt grhhterldn qlrgragrqg dpgssvffss weddvvaanl
601 dhnklpmatd engrivsprt gslldhaqrv aegrlldvha ntwrynqlia qqraiiverr
661 ntllrtvtar eelaelapkr yeelsdkvse erleticrqi mlyhldrgwa dhlayladir
721 esihlralgr qnpldefhrm avdafaslaa daieaaqqtf etanvldhep gldlsklarp
781 tstwtymvnd nplsddtlsa lslpgvfr

SEQ ID NO:6 – *SecA2* cDNA sequence of *M. tuberculosis* - nucleotides 335200-337626 of GenBank Accession BX842577

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ggcacgtgca cgtcgtgacg attaacgatt acctggcccg ccgcgatgcg gagtggatgg
gcccgctgct ggacgcgatg ggcctgacgg tcggctggat caccgcggac tcgacccctg
acgagcgccg gaccgcatat gaccgtgatg tcacctatgc ctcggtcaac gagattggct
tcgatgtact gcgcgatcag ttggtgactg atgtcaatga cctggtatcg cccaatccag
acgtggctct catcgacgaa gccgactccg tgctggtcga cgaggcgctg gtgcccctgg
tgctggccgg aaccacacat cgtgagacgc cgcggctgga gatcatccgg ctggtcgctg
agcttgttgg cgacaaggac gccgacgagt actttgccac cgattccgat aaccgcaatg
tccacttgac cgagcacggg gcacgcaaag tcgagaaagc gctcggtggc atcgacctgt
actccgagga gcacgtcggc accacactga ctgaggtcaa tgtcgcgctg cacgcgcatg
tgctcctgca acgcgacgtg cactacatcg tccgcgacga cgcggtgcac ctgatcaacg
cgtcgcgtgg ccgtatcgcg caactgcagc gctggccgga cgggttgcaa gctgcggtcg
aggccaagga aggtatcgag accacggaaa ctggggaagt gctcgacacc atcacggtgc
aggccctgat caaccggtat gcgactgtgt gcggaatgac gggaaccgcg ctggccgccg
gtgagcagct acggcagttc taccagctcg gtgtctcacc gataccaccg aacaagccaa
acatccgcga ggacgaggcc gaccgggtct acatcaccac tgcagccaag aacgacggga
tcgtcgagca catcaccgag gtgcaccaga gggggcagcc tgtgctggtc ggtacccgcg
acgtggccga atccgaggaa ctgcacgaac gcctggtgcg ccgcggtgtg cccgccgtgg
tgctcaacgc gaagaacgac gccgaggagg cccgggtcat cgccgaggcc ggcaaatacg
gcgcggtcac ggtgtcaact caaatggccg ggcgcggcac cgacatcagg ctcggcgggt
ccgacgaagc tgaccacgac agggtcgcgg aattgggcgg cctgcacgtg gtcggcactg
gccgtcacca caccgagcgg ctagacaacc agctgcgcgg tcgggccggg cgccagggag
atcccgggtc gtcggtgttt ttctcaagct gggaagacga tgtcgttgcg gccaacctcg
accacaacaa gctgccgatg gcaaccgacg aaaatggccg gattgtcagc ccgaggacgg
gtagtctgct cgaccatgcc cagcgcgttg ccgagggccg gttattggat gtgcacgcca
acacgtggcg ctacaaccag ctgatcgccc agcagcgcgc catcatcgtc gaacggcgta
acacgttgtt gcgcaccgta accgcgcgtg aggaactcgc cgaactggcg cctaagcggt
acgaggagct gtccgacaaa gtatccgagg aacgcctcga gacgatttgt cggcagatca
tgctgtatca cctcgaccgt ggctgggccg atcacctggc gtatctggcc gacatccggg
agagcatcca tctacgcgcg ctgggccggc agaacccact cgacgagttt caccggatgg
ctgtggacgc gttcgcgtcg ctggccgccg acgccatcga ggcggctcaa cagacgttcg
aaaccgcgaa cgtccttgac cacgagccgg ggctggacct gtccaaactg gcccggccga
cgtcgacatg gacctacatg gtcaatgaca acccactgtc cgatgacacg ctttctgccc
tcagtctgcc cggggtgttc cgctga

What is claimed is:

1. A recombinant mycobacterium having a mutation in an *nlaA* gene, wherein the mutation increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium.

2. The mycobacterium of claim 1, wherein the *nlaA* gene without the mutation encodes a protein that is at least about 85% homologous to SEQ ID NO:1.

3. The mycobacterium of claim 1, wherein the *nlaA* gene without the mutation encodes a protein that is at least about 99% homologous to SEQ ID NO:1.

4. The mycobacterium of claim 1, wherein the *nlaA* gene without the mutation encodes a protein having the amino acid sequence of SEQ ID NO:1.

5. The mycobacterium of claim 1, wherein the mutation is a deletion.

6. The mycobacterium of claim 5, wherein the deletion is a deletion of the entire *nlaA* gene (Δ*nlaA*).

7. A recombinant mycobacterium having a mutation in a *nuoG* gene, wherein the mutation increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium.

8. The mycobacterium of claim 7, wherein the *nuoG* gene without the mutation encodes a protein that is at least about 85% homologous to SEQ ID NO:3.

9. The mycobacterium of claim 7, wherein the *nuoG* gene without the mutation encodes a protein that is at least about 99% homologous to SEQ ID NO:3.

10. The mycobacterium of claim 7, wherein the *nuoG* gene without the mutation encodes a protein having the amino acid sequence of SEQ ID NO:3.

11. The mycobacterium of claim 7, wherein the mutation is a deletion.

12. The mycobacterium of claim 11, wherein the deletion is a deletion of the entire *nuoG* gene (Δ*nuoG*).

13. The mycobacterium of any one of claims 1-12, further comprising a second mutation, wherein the mycobacterium exhibits attenuated virulence in a mammal when compared to the mycobacterium without the second mutation.

14. The mycobacterium of claim 13, wherein the second mutation is a deletion in at least a portion of an RD1 region, or a deletion in a gene controlling production of a vitamin or an amino acid.

15. The mycobacterium of any one of claims 1-14 wherein the mycobacterium is *M. smegmatis*, *M. bovis*, *M. avium*, *M. phlei*, *M. fortuitum*, *M. lufu*, *M. paratuberculosis*, *M. habana*, *M. scrofulacium*, *M. intracellulare*, *M. tuberculosis*, or *M. kansasii*.

16. The mycobacterium of claim 15, wherein the mycobacterium is an *M. bovis* BCG.

17. The mycobacterium of claim 1, wherein the mycobacterium is *M. tuberculosis*.

18. The mycobacterium of any one of claims 1-17, further comprising a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell.

19. The mycobacterium of claim 18, wherein the gene encodes an antigen of a mammalian pathogen.

20. The mycobacterium of claim 19, wherein the pathogen is a virus.

21. The mycobacterium of claim 19, wherein the pathogen is a bacterium.

22. The mycobacterium of claim 19, wherein the pathogen is a eukaryotic parasite.

23. The mycobacterium of claim 19, wherein the mammalian pathogen is a human pathogen.

24. An isolated and purified nlaA protein from a mycobacterium, having an amino acid sequence at least 85% identical to SEQ ID NO:1, wherein the nlaA protein prevents the mycobacterium from inducing apoptosis in a mammalian macrophage.

25. The nlaA protein of claim 24, wherein the amino acid sequence is at least 99% identical to SEQ ID NO:1.

26. The nlaA protein of claim 24, wherein the amino acid sequence is SEQ ID NO:1.

27. The nlaA protein of claim 24, wherein the protein was expressed recombinantly.

28. An isolated and purified nuoG protein from a mycobacterium, having an amino acid sequence at least 85% identical to SEQ ID NO:3, wherein the nlaA protein prevents the mycobacterium from inducing apoptosis in a mammalian macrophage.

29. The nuoG protein of claim 28, wherein the amino acid sequence is at least 99% identical to SEQ ID NO:3.

30. The nuoG protein of claim 28, wherein the amino acid sequence is SEQ ID NO:3.

31. The nuoG protein of claim 28, wherein the protein was expressed recombinantly.

32. An isolated and purified nucleic acid comprising a recombinant *nlaA* gene having a nucleotide sequence at least 85% identical to SEQ ID NO:2.

33. The nucleic acid of claim 32, wherein the nucleotide sequence is at least 99% identical to SEQ ID NO:2.

34. The nucleic acid of claim 32, wherein the nucleotide sequence is SEQ ID NO:2.

35. The nucleic acid of claim 32, which is a vector capable of replication and/or expression of the nlaA protein encoded by the recombinant *nlaA* gene when transfected into a mycobacterium.

36. An isolated and purified nucleic acid comprising a recombinant *nuoG* gene having a nucleotide sequence at least 85% identical to SEQ ID NO:4.

37. The nucleic acid of claim 36, wherein the nucleotide sequence is at least 99% identical to SEQ ID NO:4.

38. The nucleic acid of claim 36, wherein the nucleotide sequence is SEQ ID NO:4.

39. The nucleic acid of claim 36, which is a vector capable of replication and/or expression of the nuoG protein encoded by the recombinant *nuoG* gene when transfected into a mycobacterium.

40. A method of inducing an immune response in a mammal, the method comprising inoculating the mammal with the mycobacterium of any one of claims 1-23.

41. The method of claim 40, wherein the mycobacterium comprises a mutation in an *nlaA* gene, wherein the mutation increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium.

42. The method of claim 40, wherein the mycobacterium comprises a mutation in a *nuoG* gene, wherein the mutation increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium.

43. The method of claim 40, wherein the mycobacterium comprises a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell.

44. The method of claim 43, wherein the gene encodes an antigen of a mammalian pathogen.

45. The method of claim 44, wherein the antigen is of a human pathogen.

46. The method of claim 44, wherein the pathogen is a virus.

47. The method of claim 44, wherein the pathogen is a bacterium.

48. The method of claim 44, wherein the pathogen is a eukaryotic parasite.

49. The method of claim 40, wherein the mycobacterium is an *M. tuberculosis* or an *M. bovis*.

50. The method of claim 40, wherein the inoculation gives the mammal increased immunity to a virulent *M. tuberculosis*.

51. A method of making a recombinant mycobacterium, the method comprising eliminating expression of the *nlaA* gene in the mycobacterium.

52. The method of claim 51, wherein expression of the *nlaA* gene is eliminated by specialized transduction.

53. A method of making a recombinant mycobacterium, the method comprising eliminating expression of the *nuoG* gene in the mycobacterium.

54. The method of claim 53, wherein expression of the *nuoG* gene is eliminated by specialized transduction.

55. The method of any one of claims 51-54, further comprising eliminating expression of a second gene.

56. The method of claim 55, wherein the mycobacterium exhibits attenuated virulence in a mammal when compared to the same mycobacterium expressing the second gene.

57. The method of claim 56, wherein the second gene is a portion of an RD1 region, or a gene controlling production of a vitamin or an amino acid.

58. The method of claim 55, wherein eliminating expression of the second gene increases the ability of the mycobacterium to induce apoptosis of a mammalian macrophage infected by the mycobacterium.

59. The method of any one of claims claim 51-58, wherein the mycobacterium further comprises a recombinant gene operably linked to a promoter that directs expression of the gene when the mycobacterium infects a mammalian cell.

60. The method of any one of claims 51-59, wherein the mycobacterium is *M. smegmatis*, *M. bovis*, *M. avium*, *M. phlei*, *M. fortuitum*, *M. lufu*, *M. paratuberculosis*, *M. habana*, *M. scrofulacium*, *M. intracellulare*, *M. tuberculosis*, or *M. kansasii*.

61. The method of claim 60, wherein the mycobacterium is *M. tuberculosis*.

62. The method of claim 60, wherein the mycobacterium is *M. bovis* BCG.

FIG. 1

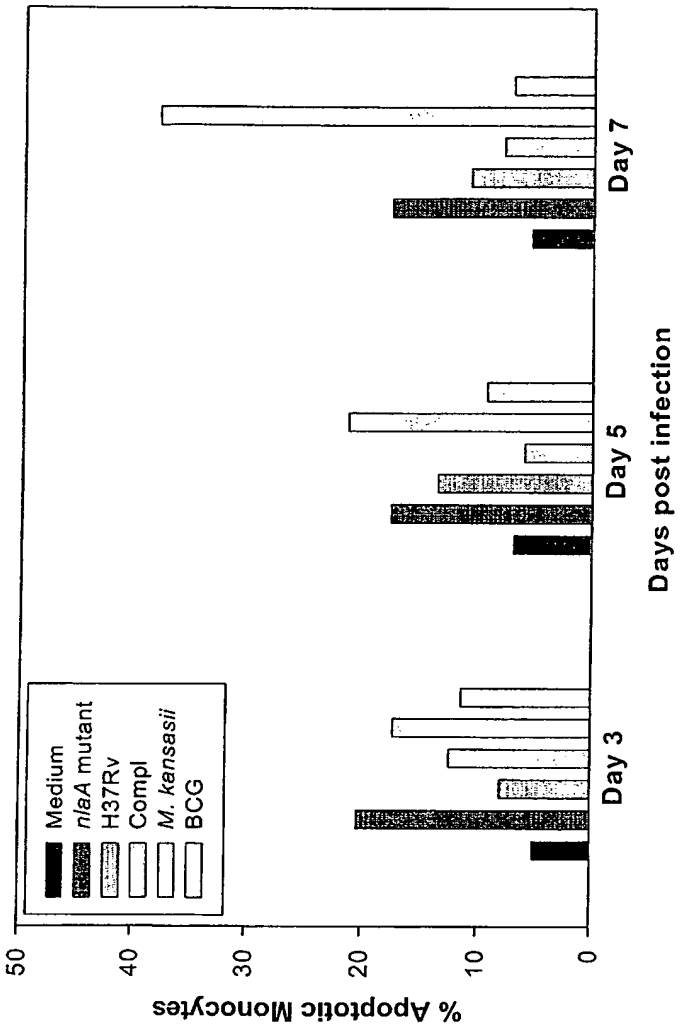

% Apoptotic Monocytes
0
10
20
30
40
50
Day 3
Day 5
Day 7
Days post infection
Medium
nlaA mutant
H37Rv
Compl
M. kansasii
BCG

FIG. 2

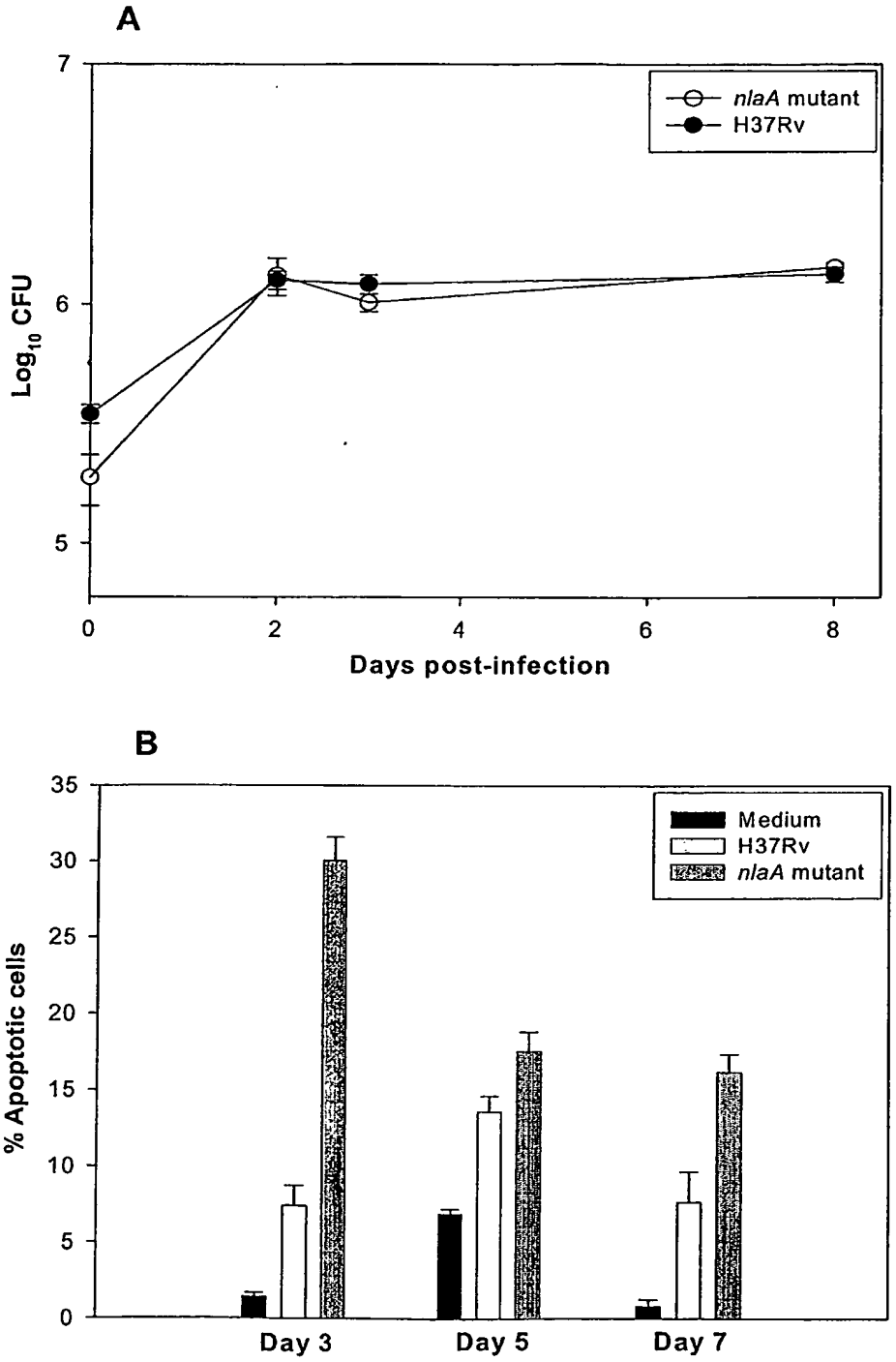
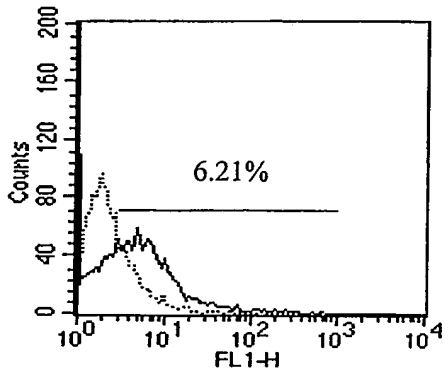
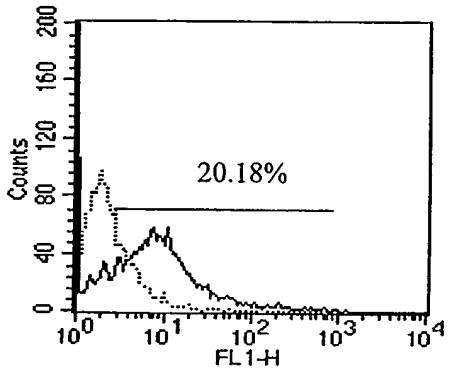

A
7
6
5
Log10 CFU
nlaA mutant
H37Rv
0
2
4
6
8
Days post-infection
B
35
30
25
20
15
10
5
0
% Apoptotic cells
Medium
H37Rv
nlaA mutant
Day 3
Day 5
Day 7

FIG. 3

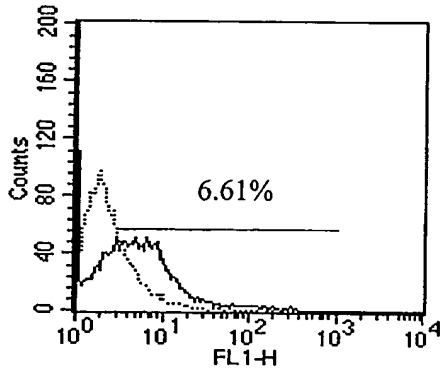


M. tuberculosis H37Rv

B



M. tuberculosis Δ*nlaA*



M. tuberculosis complemented strain

FIG. 4

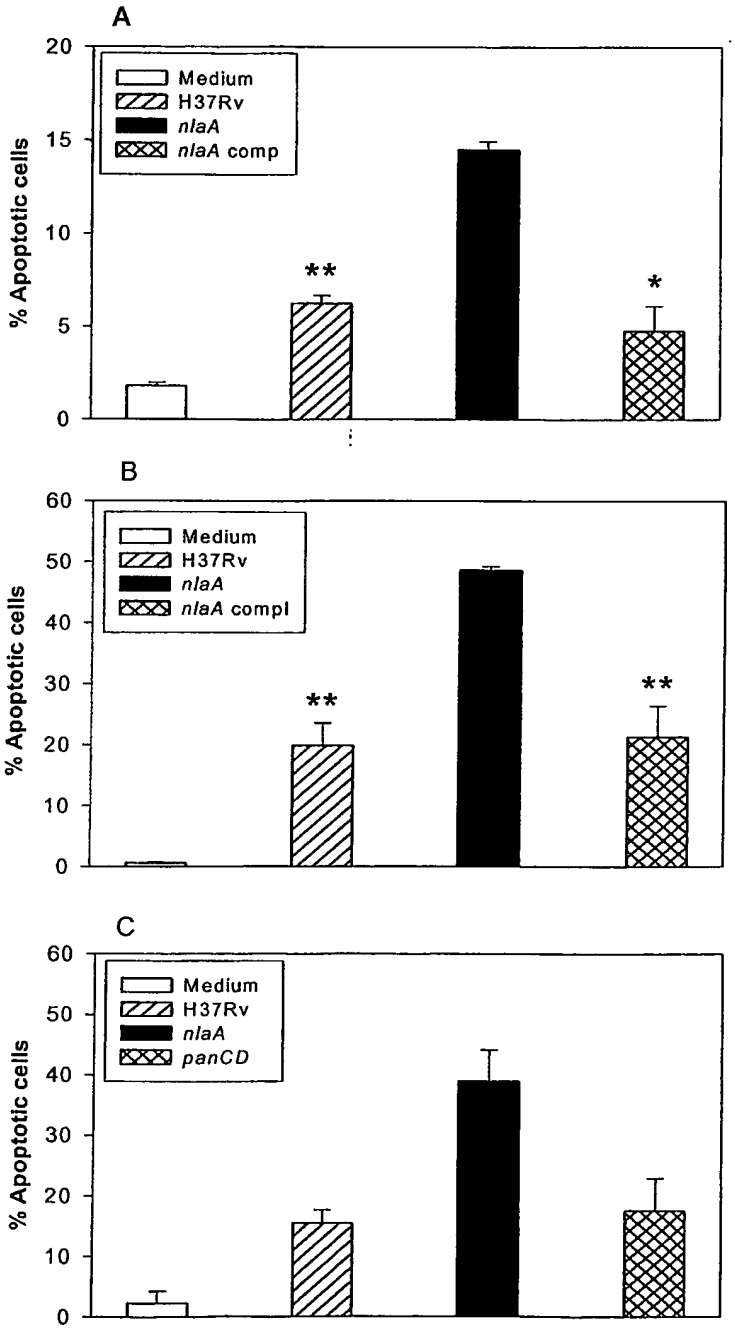

A
% Apoptotic cells
20
15
10
5
0
Medium
H37Rv
nlaA
nlaA comp
**
*
B
% Apoptotic cells
60
50
40
30
20
10
0
Medium
H37Rv
nlaA
nlaA compl
**
**
C
% Apoptotic cells
60
50
40
30
20
10
0
Medium
H37Rv
nlaA
panCD

FIG. 5

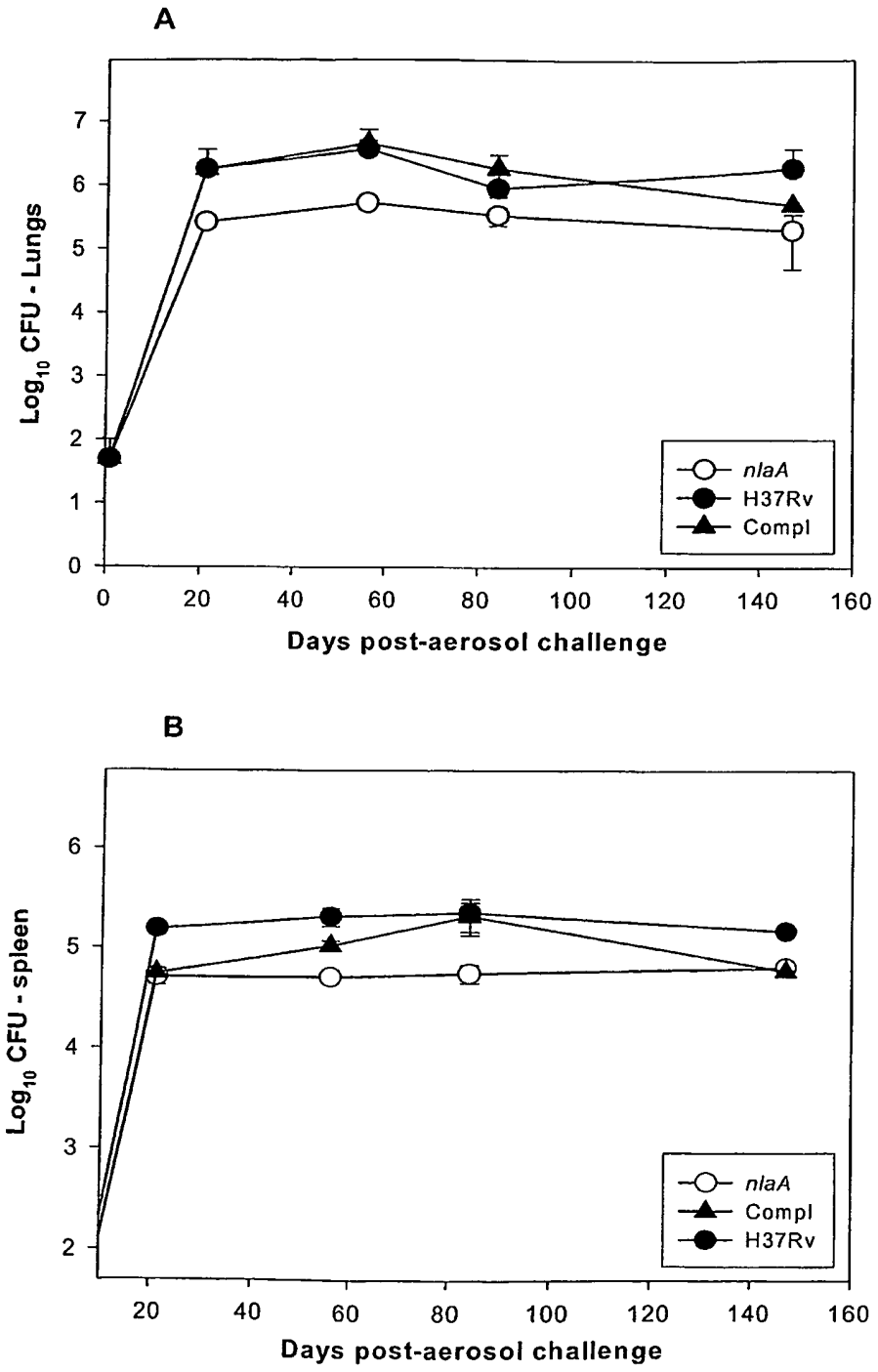

A
Log10 CFU - Lungs
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120
140
160
Days post-aerosol challenge
nlaA
H37Rv
Compl
B
Log10 CFU - spleen
2
3
4
5
6
20
40
60
80
100
120
140
160
Days post-aerosol challenge
nlaA
Compl
H37Rv

FIG. 6

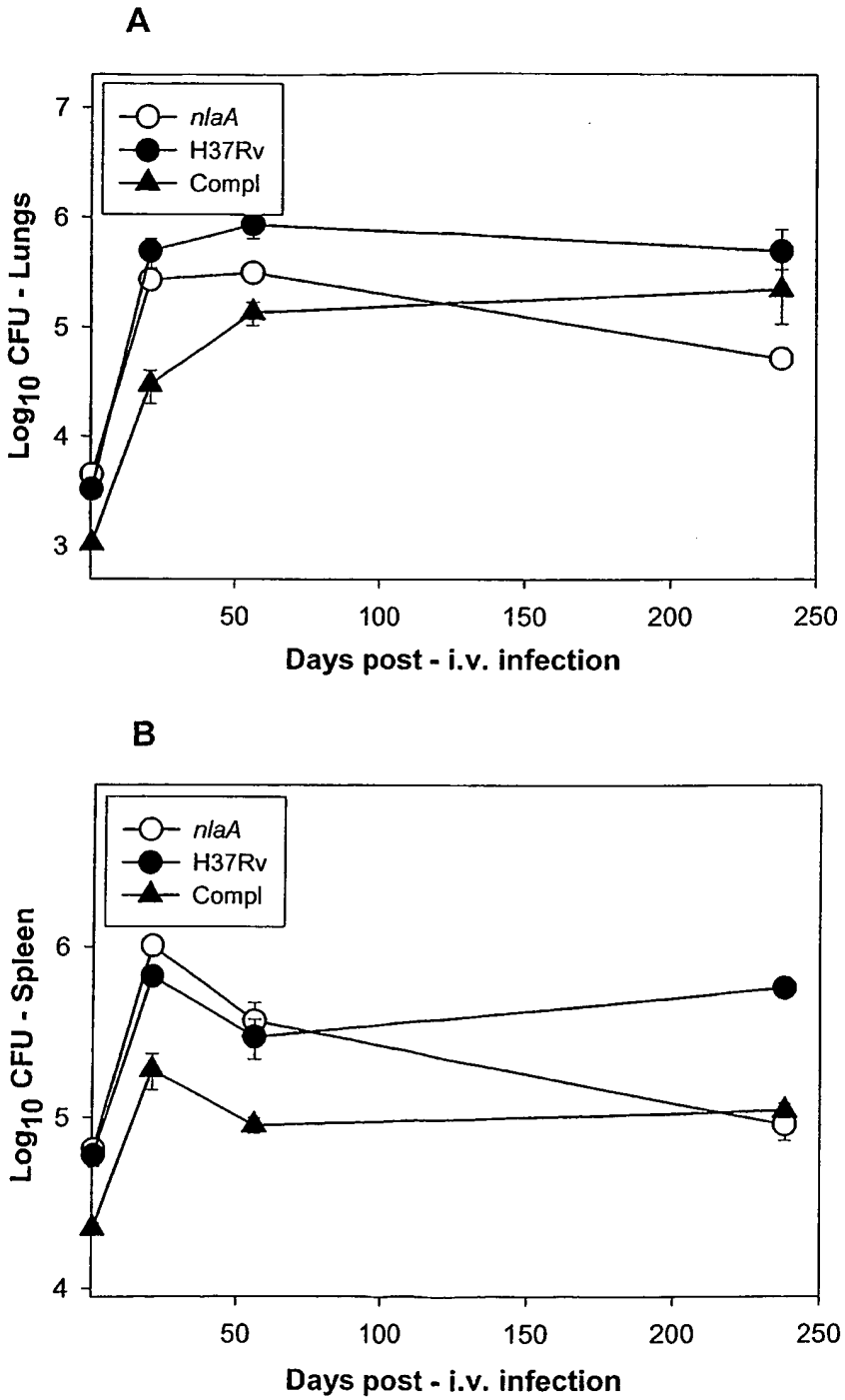

A
nlaA
H37Rv
Compl
Log10 CFU - Lungs
7
6
5
4
3
50
100
150
200
250
Days post - i.v. infection
B
nlaA
H37Rv
Compl
Log10 CFU - Spleen
6
5
4
50
100
150
200
250
Days post - i.v. infection

FIG. 7

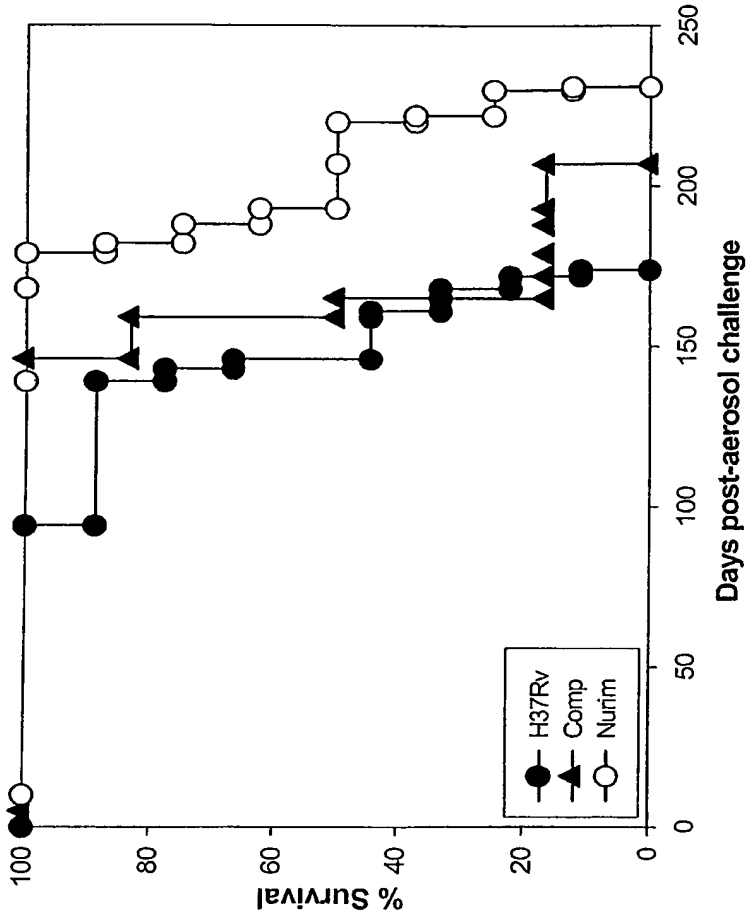

% Survival
100
80
60
40
20
0
0
50
100
150
200
250
Days post-aerosol challenge
H37Rv
Comp
Nurim

FIG. 8

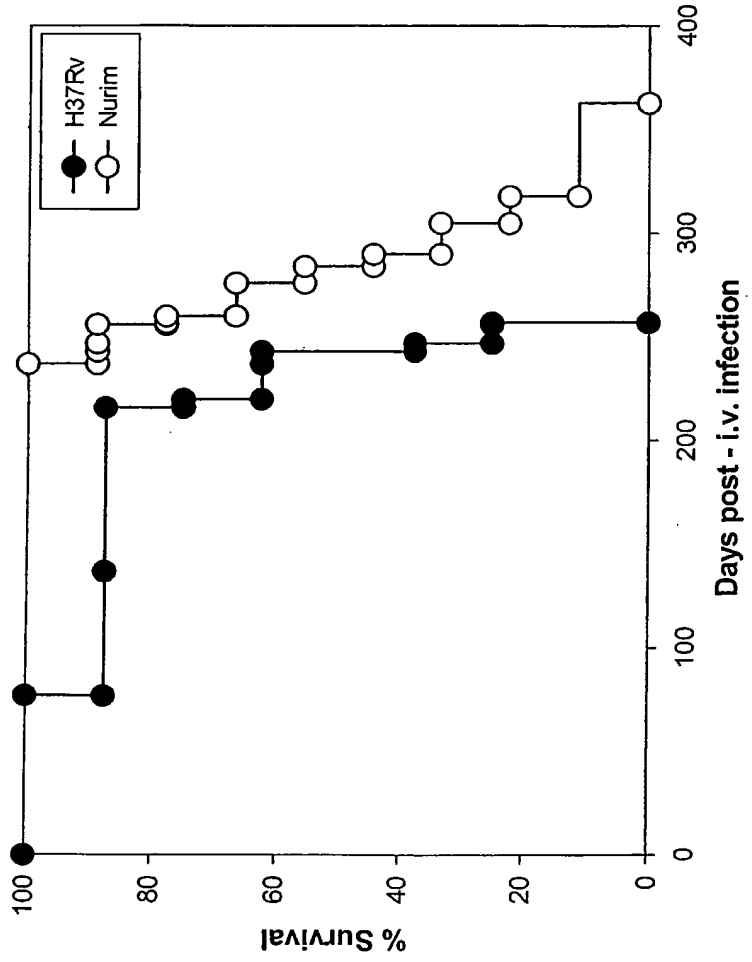

H37Rv
Nurim
% Survival
100
80
60
40
20
0
0
100
200
300
400
Days post - i.v. infection

FIG. 9

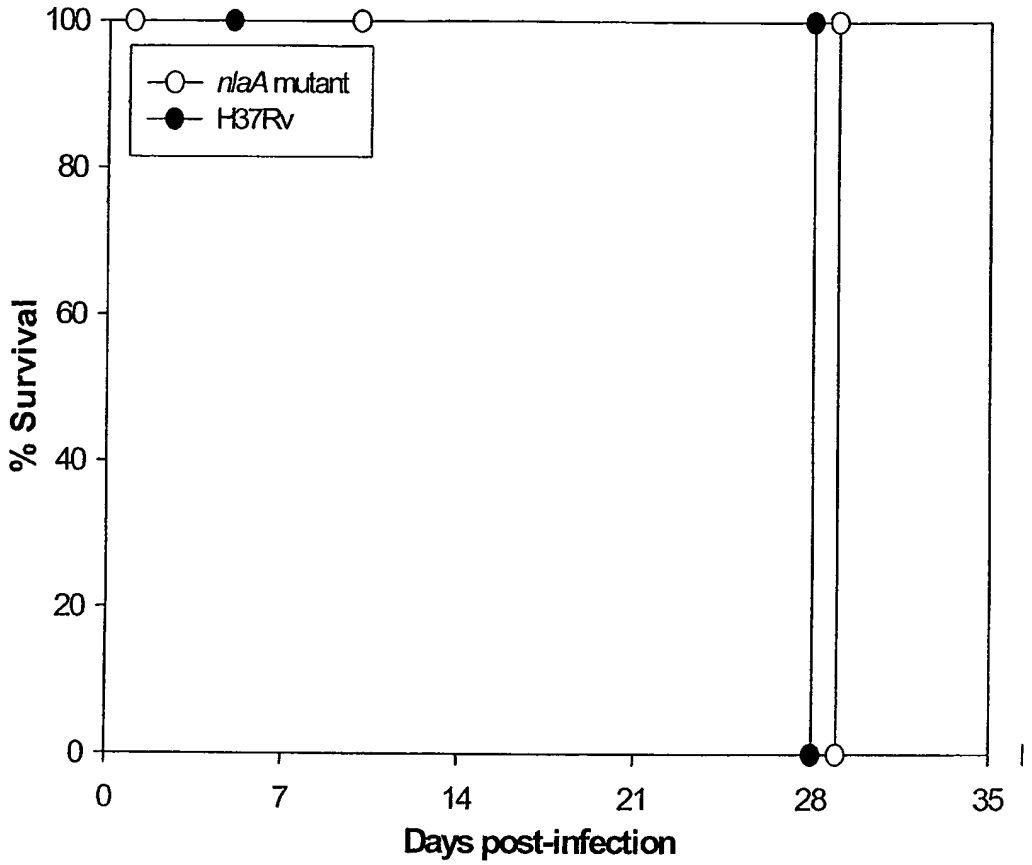

100
80
60
40
20
0
% Survival
nlaA mutant
H37Rv
0
7
14
21
28
35
Days post-infection

FIG. 10

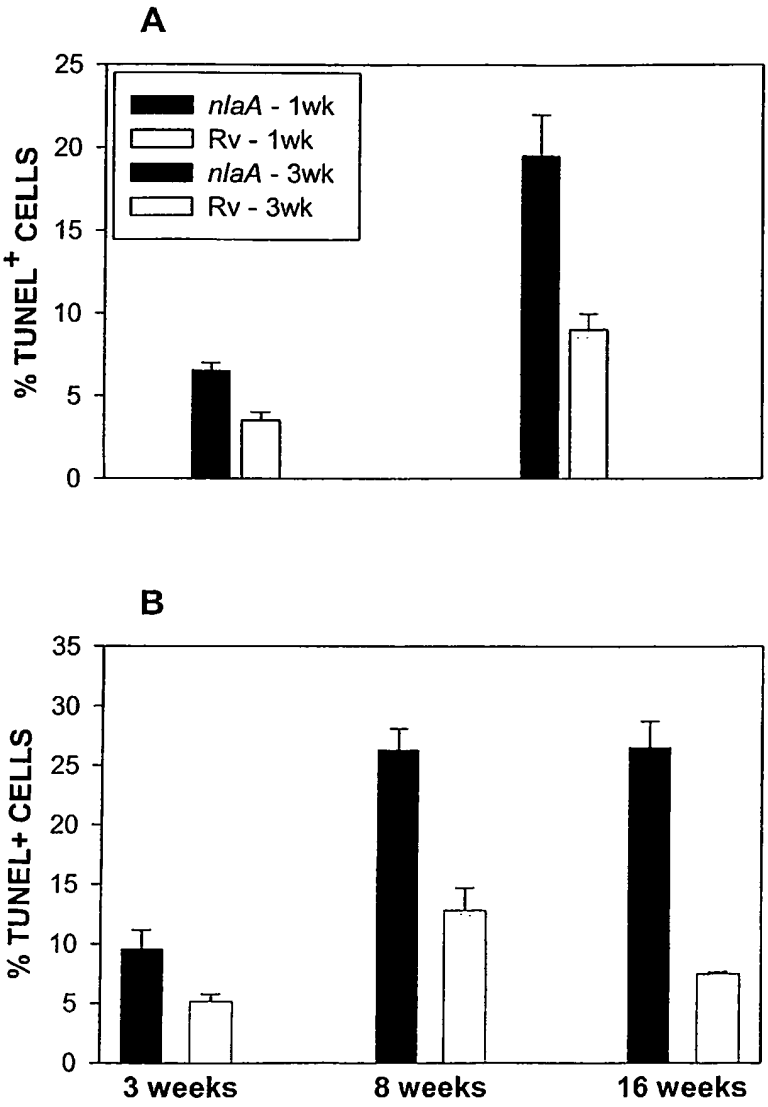

A
25
20
15
10
5
0
% TUNEL+ CELLS
nlaA - 1wk
Rv - 1wk
nlaA - 3wk
Rv - 3wk
B
35
30
25
20
15
10
5
0
% TUNEL+ CELLS
3 weeks
8 weeks
16 weeks

FIG. 11

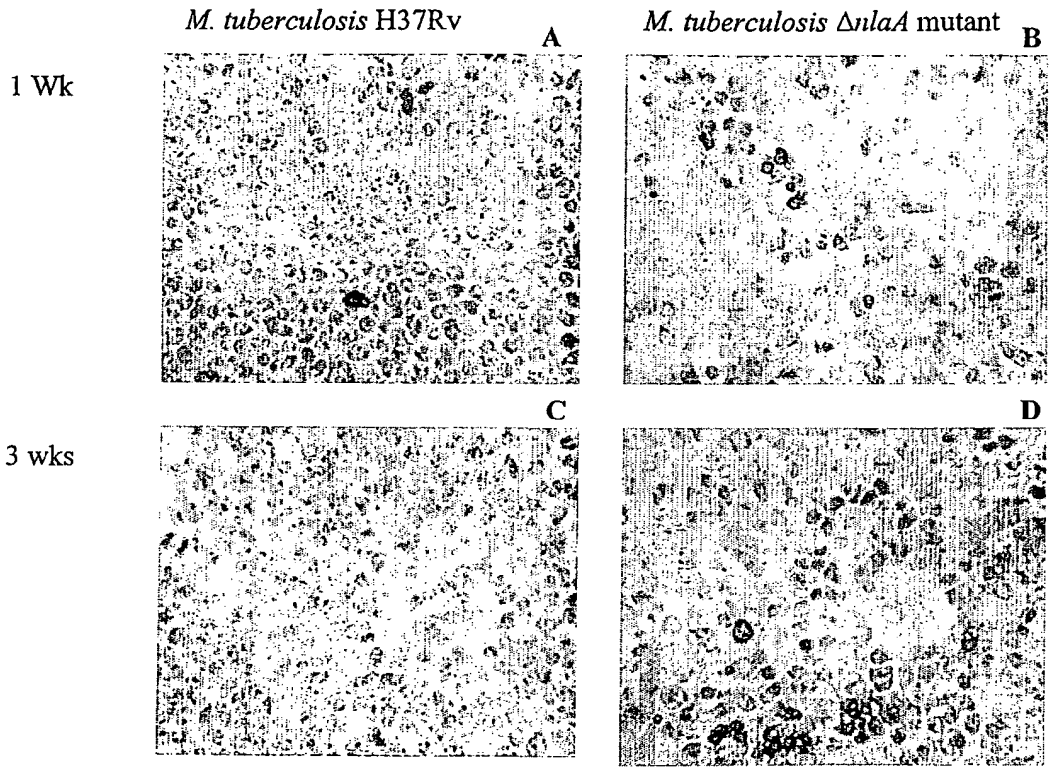

M. tuberculosis H37Rv
M. tuberculosis ΔnlaA mutant
A
B
C
D
1 Wk
3 wks

FIG. 12

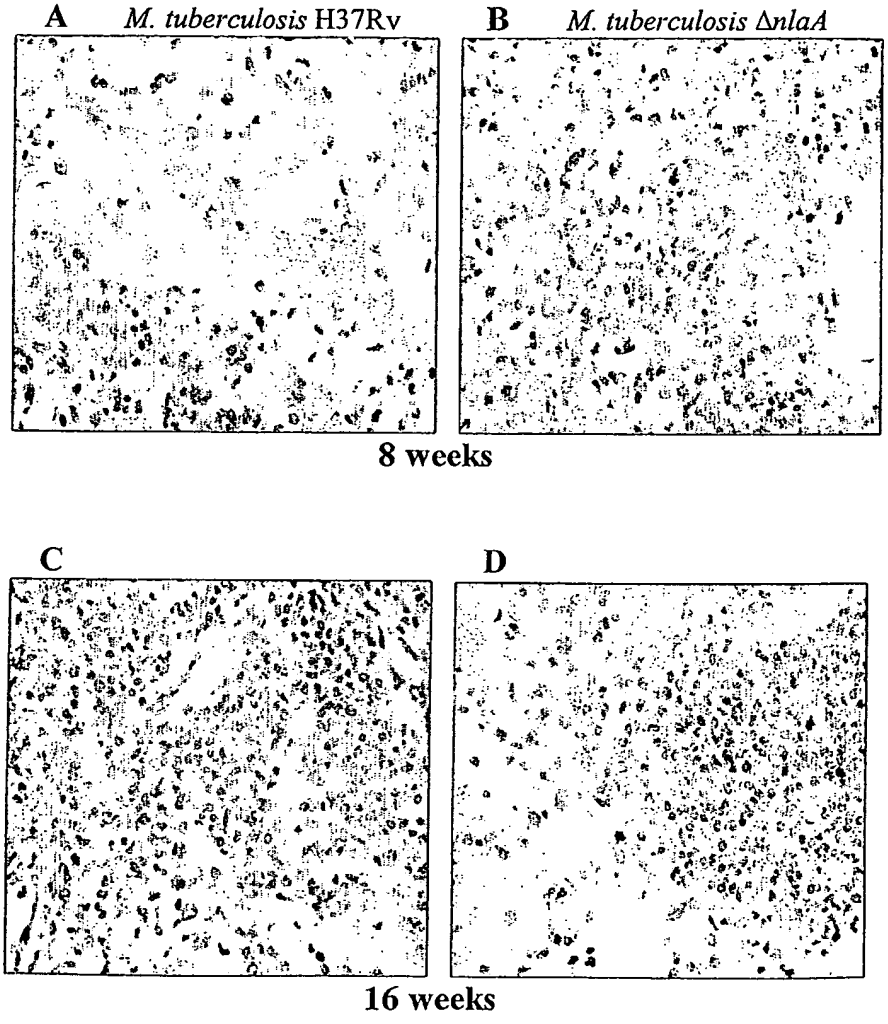

A
M. tuberculosis H37Rv
B
M. tuberculosis ΔnlaA
8 weeks
C
D
16 weeks

FIG. 13

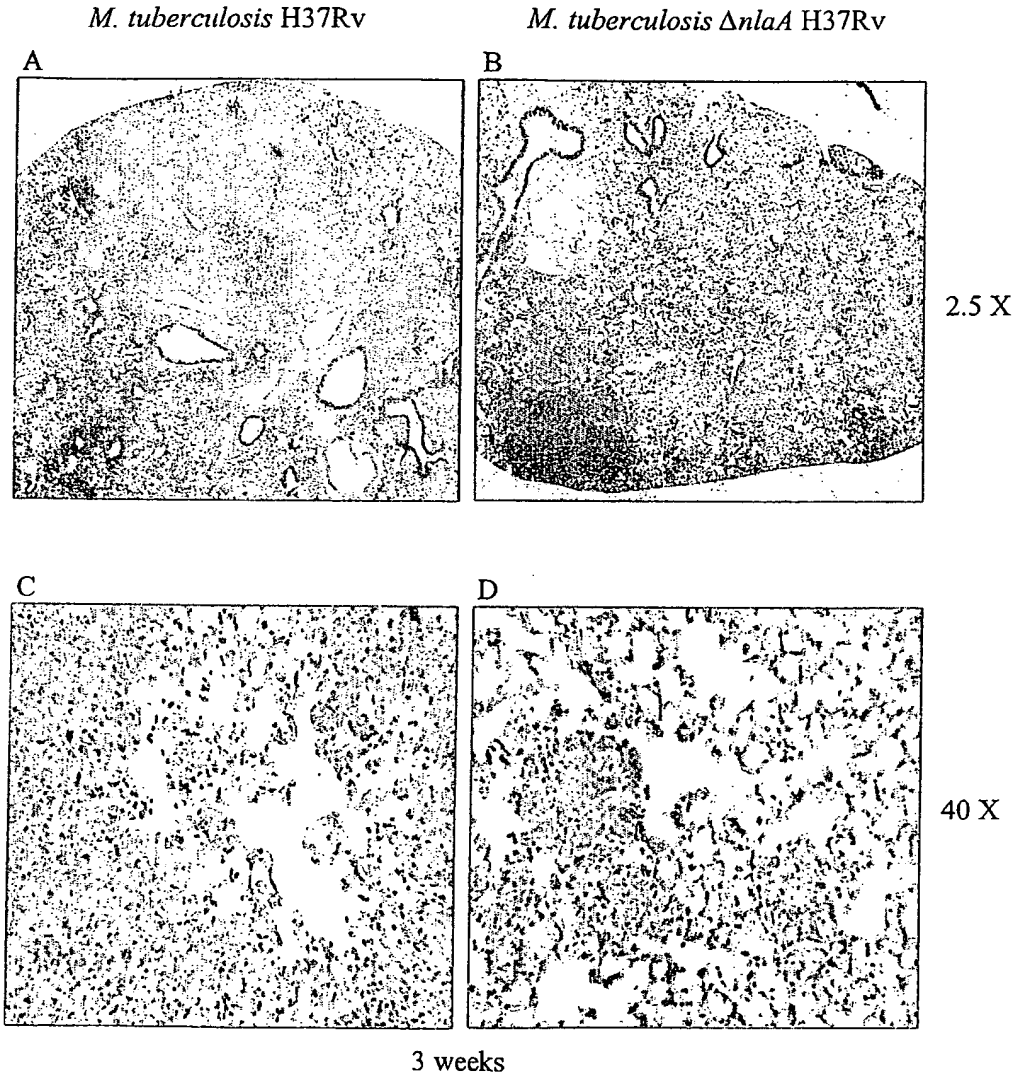

M. tuberculosis H37Rv
M. tuberculosis ΔnlaA H37Rv
A
B
2.5 X
C
D
40 X
3 weeks

FIG. 14

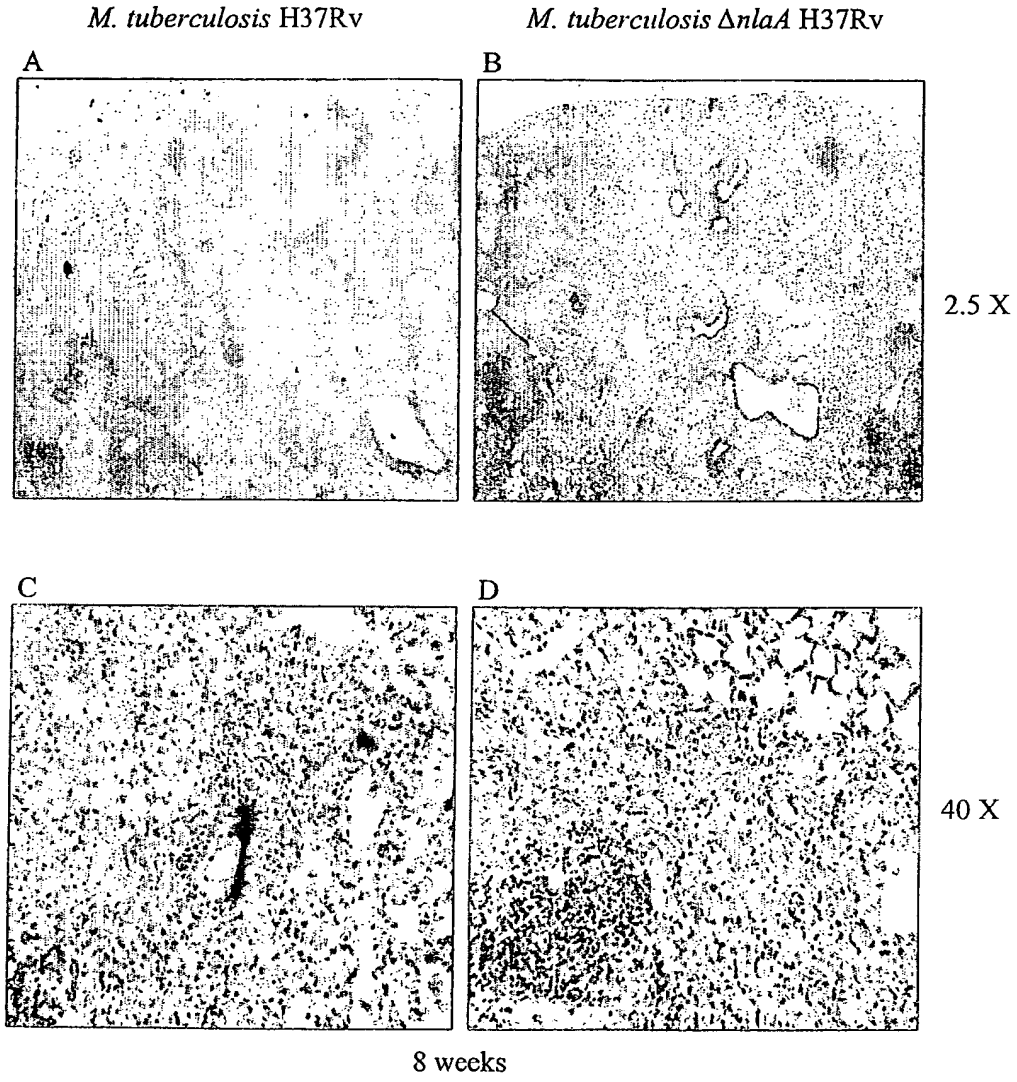

M. tuberculosis H37Rv
M. tuberculosis ΔnlaA H37Rv
A
B
2.5 X
C
D
40 X
8 weeks

FIG. 15

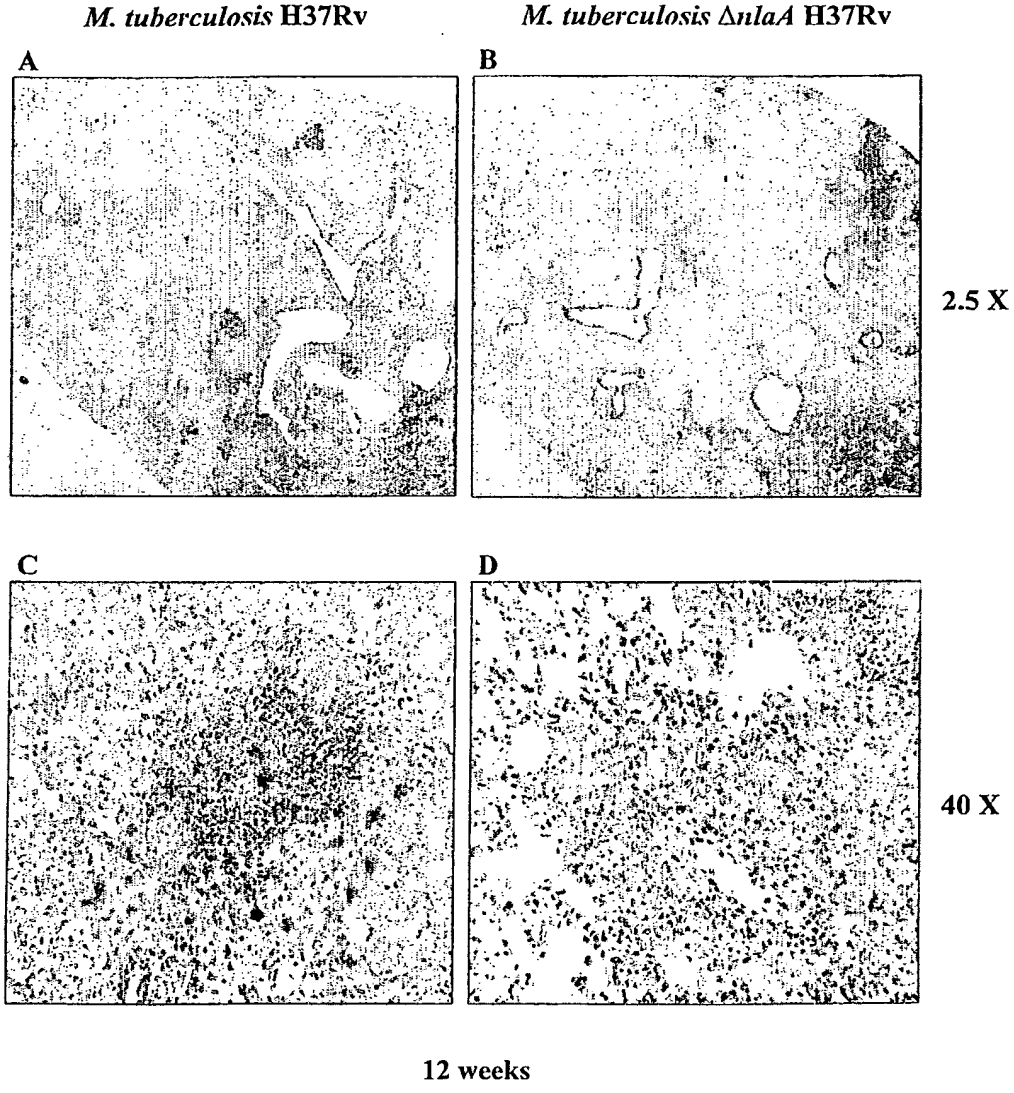

M. tuberculosis H37Rv
M. tuberculosis ΔnlaA H37Rv
A
B
2.5 X
C
D
40 X
12 weeks

FIG. 16

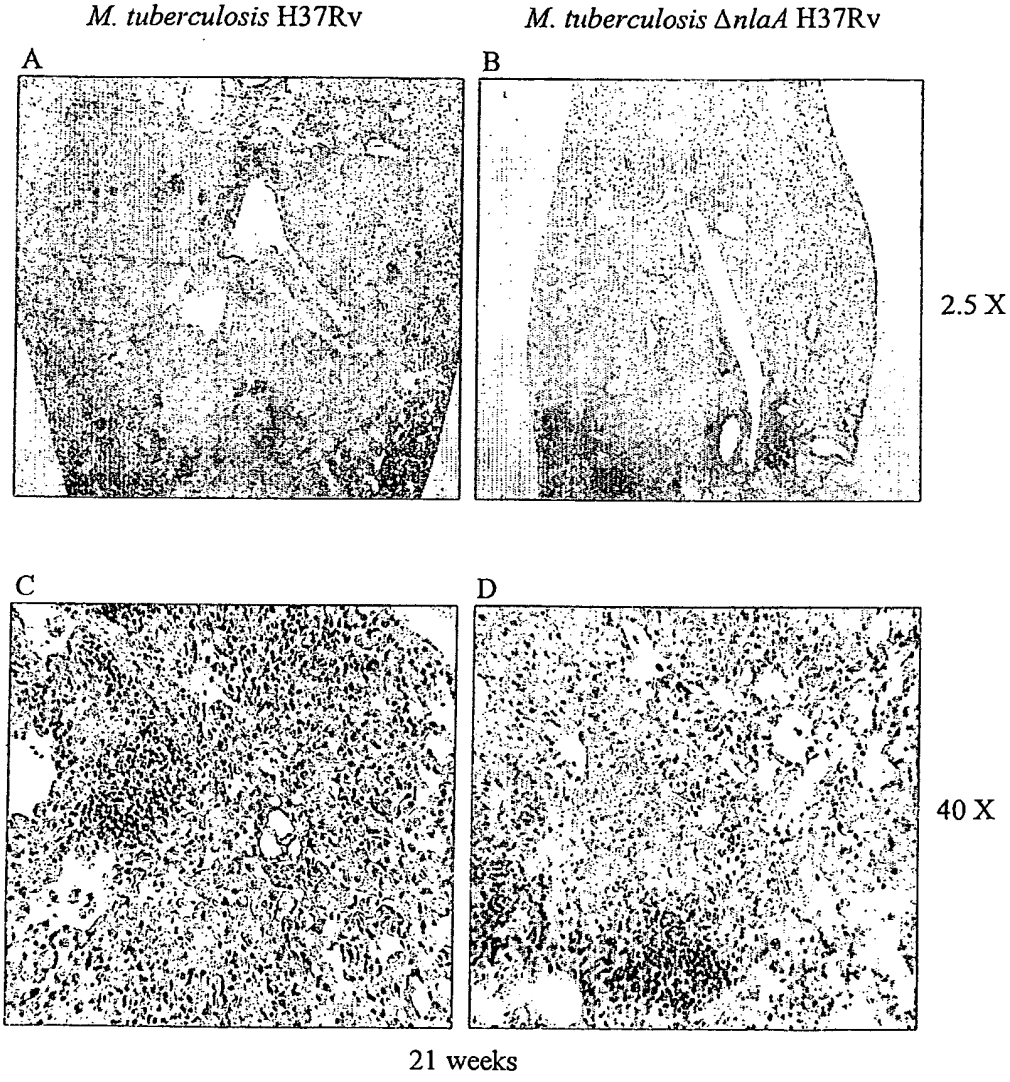

M. tuberculosis H37Rv
M. tuberculosis ΔnlaA H37Rv
A
B
2.5 X
C
D
40 X
21 weeks

FIG. 17

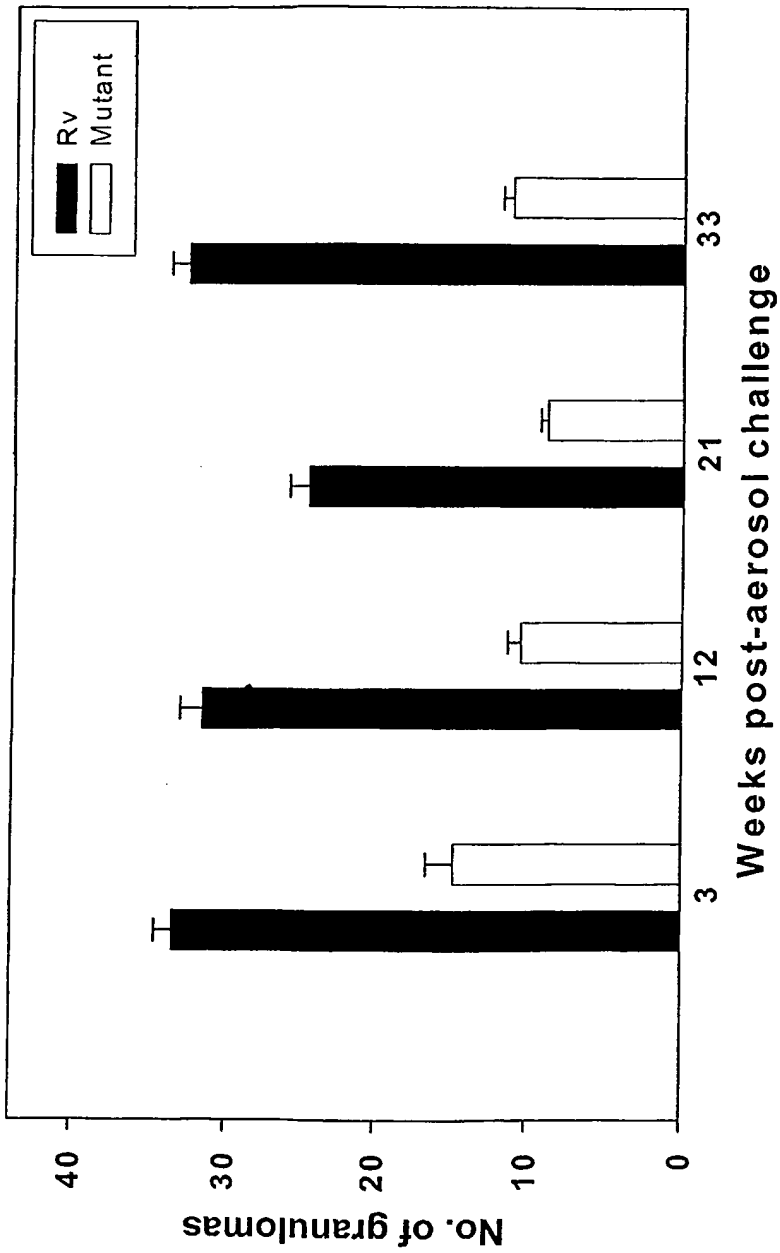

Rv
Mutant
No. of granulomas
0
10
20
30
40
3
12
21
33
Weeks post-aerosol challenge

FIG. 18

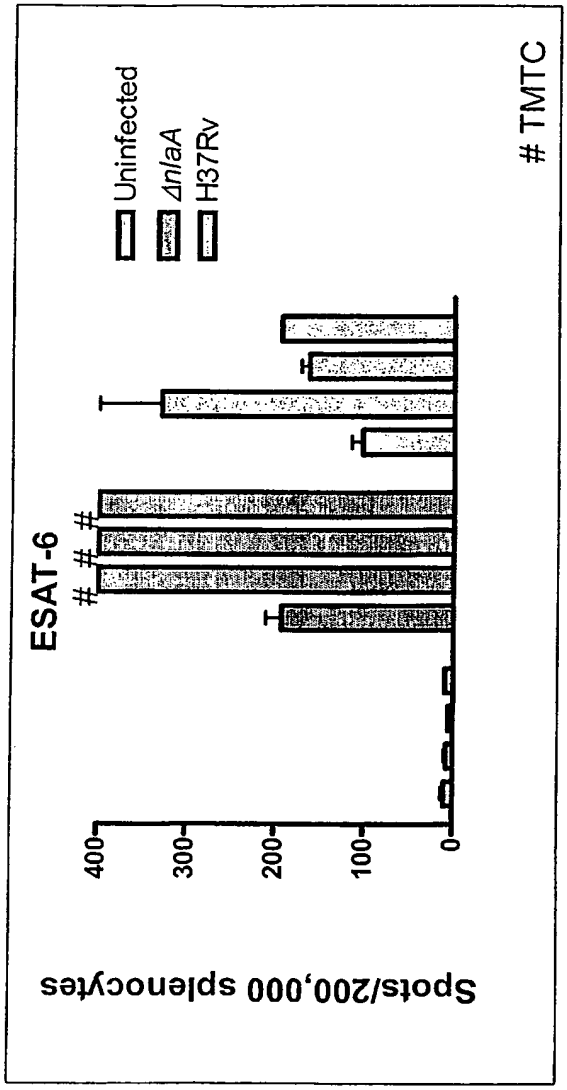

Spots/200,000 splenocytes
400
300
200
100
0
ESAT-6
# # #
Uninfected
ΔnlaA
H37Rv
TMTC

FIG. 19

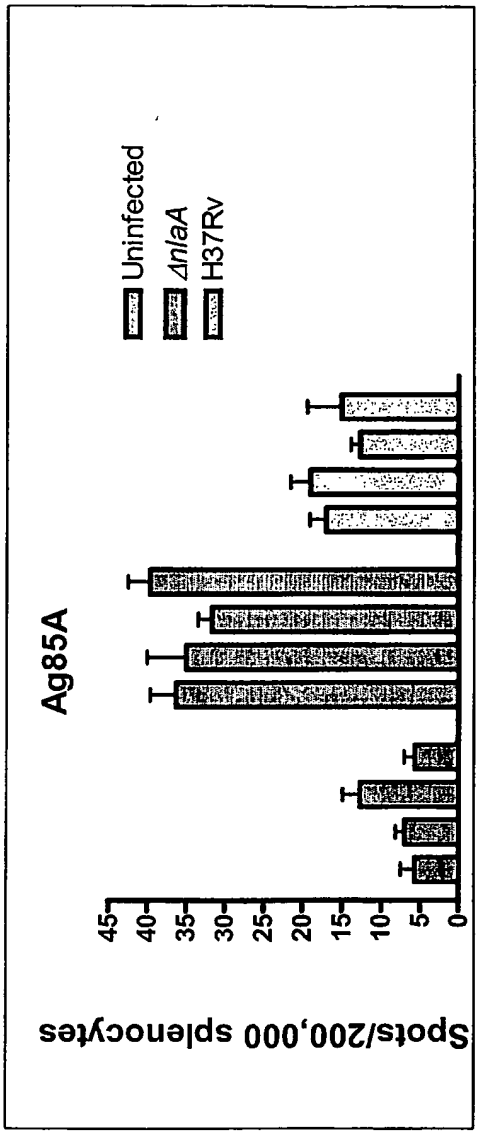

Spots/200,000 splenocytes
45
40
35
30
25
20
15
10
5
0
Ag85A
Uninfected
ΔnlaA
H37Rv

FIG. 20

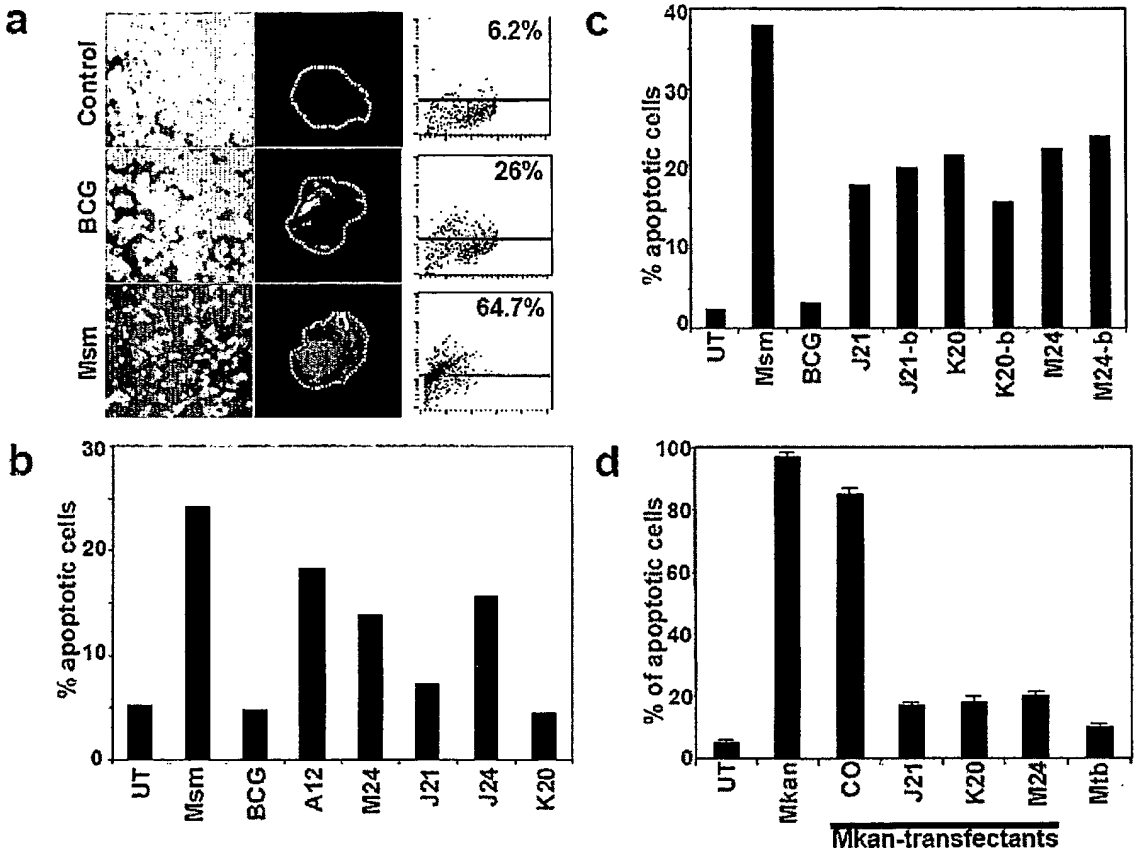

a
Control
BCG
Msm
6.2%
26%
64.7%
c
% apoptotic cells
0
10
20
30
40
UT
Msm
BCG
J21
J21-b
K20
K20-b
M24
M24-b
b
% apoptotic cells
0
10
20
30
UT
Msm
BCG
A12
M24
J21
J24
K20
d
% of apoptotic cells
0
20
40
60
80
100
UT
Mkan
CO
J21
K20
M24
Mtb
Mkan-transfectants

FIG. 21

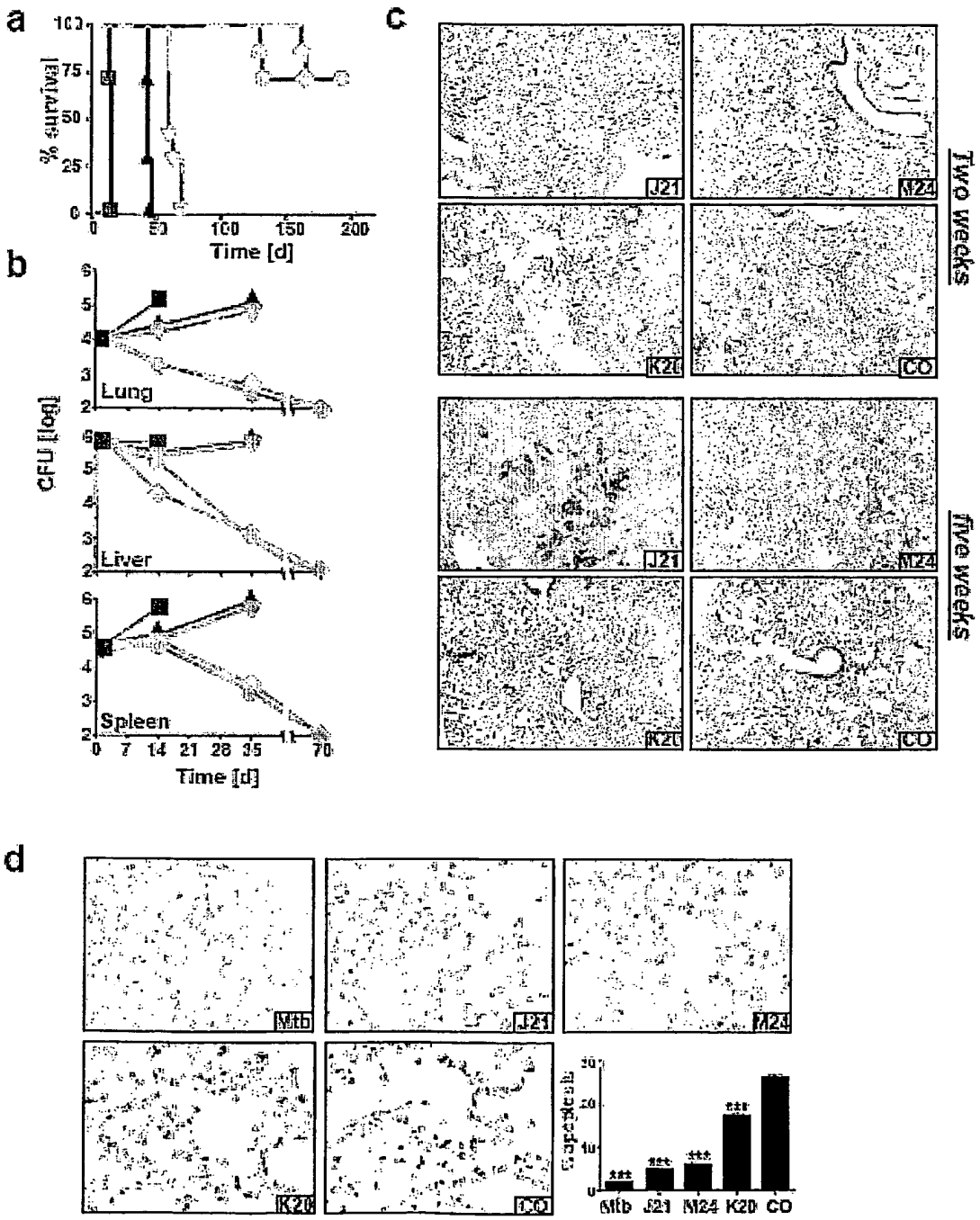

a
% survival
Time [d]
b
CFU [log]
Lung
Liver
Spleen
Time [d]
c
J21
M24
K20
CO
Two weeks
five weeks
d
Mtb
J21
M24
K20
CO
Mtb J21 M24 K20 CO

FIG. 22

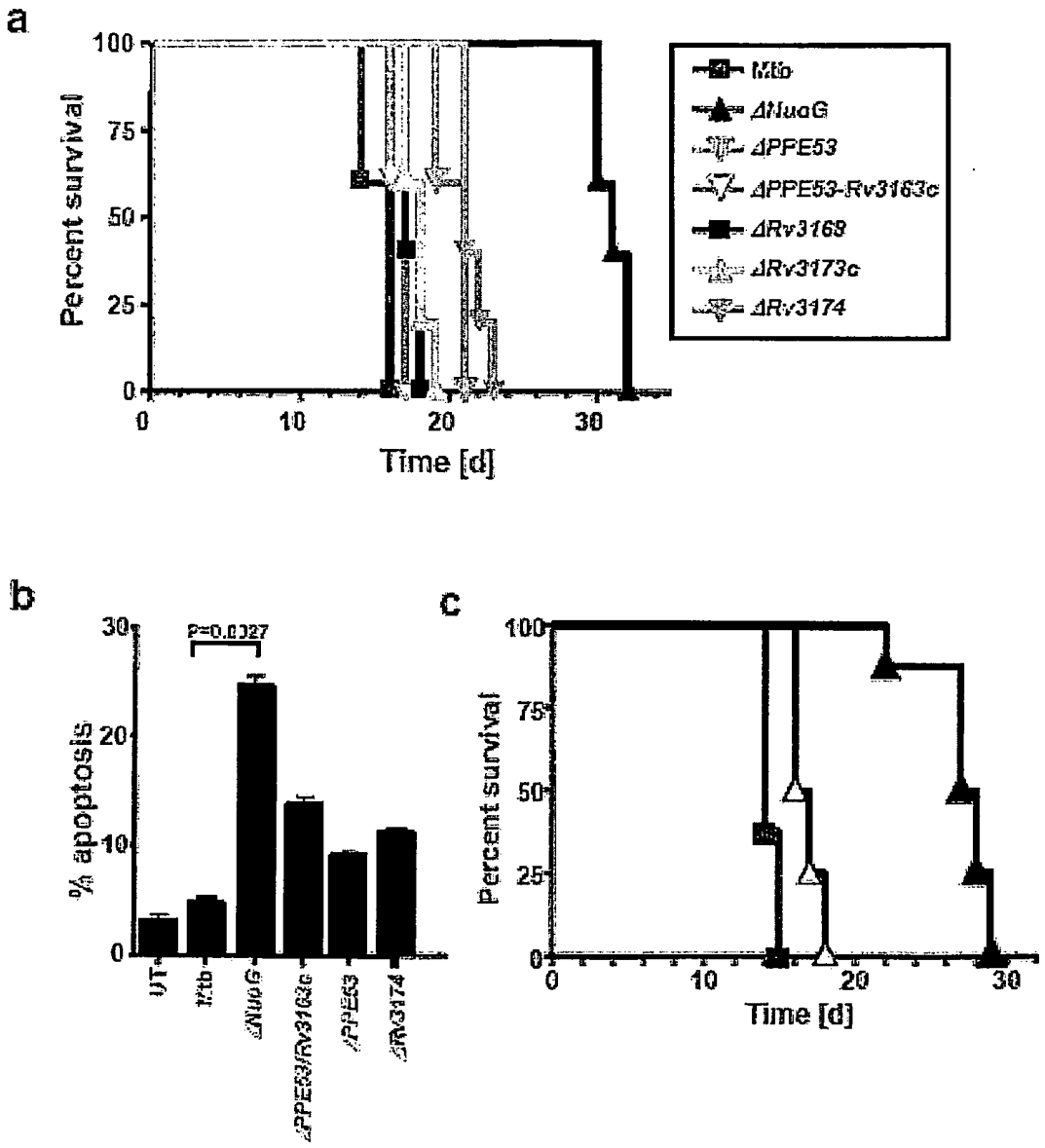

a
Percent survival
Time [d]
Mtb
ΔNuoG
ΔPPE53
ΔPPE53-Rv3163c
ΔRv3168
ΔRv3173c
ΔRv3174
b
% apoptosis
P=0.0327
UT
Mtb
ΔNuoG
ΔPPE53/Rv3163c
ΔPPE53
ΔRv3174
c
Percent survival
Time [d]

FIG. 23

Screen (method)	Clones screened/selected
1st screen (microscopy)	312/52
2nd screen (flow cytometry, PI-staining)	52/16
3rd screen (flow cytometry, PI-staining)	16/12
4th screen (flow cytometry, TUNEL assay)	12/4

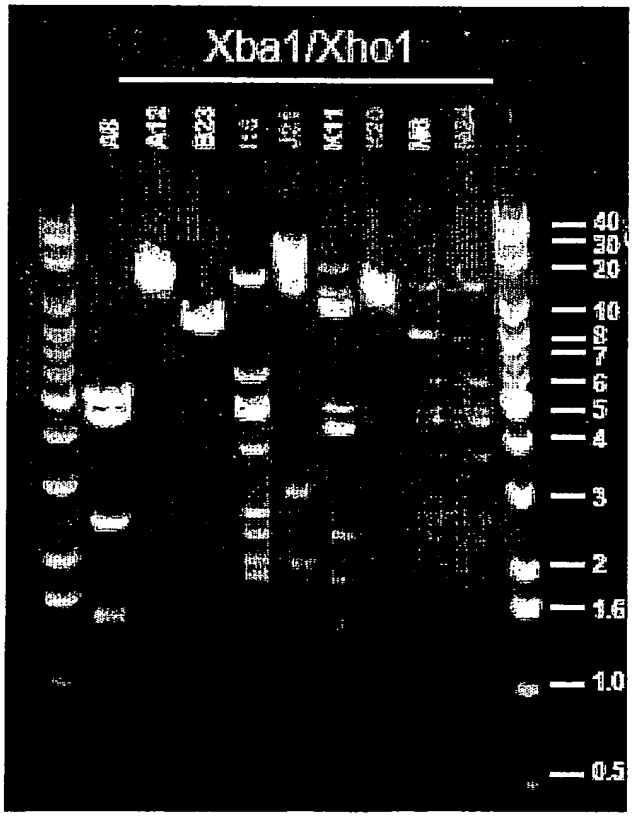


FIG. 24

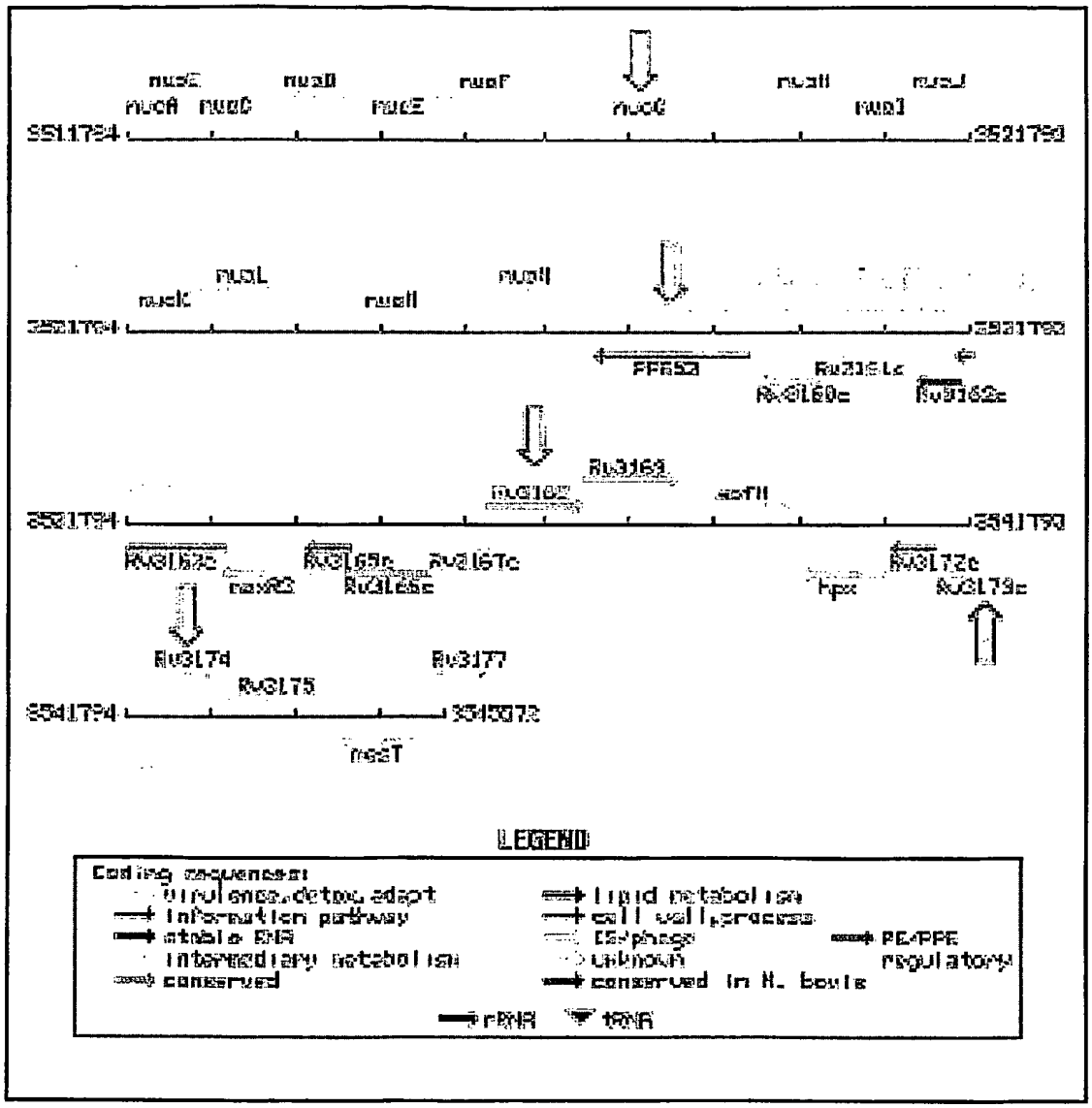

Rv3162c
Rv3163
Rv3172c
Rv3174
Rv3175
Rv3177
LEGEND
Lipid metabolism
cell wall process
PE/PPE
regulatory
conserved
rRNA
tRNA

FIG. 25

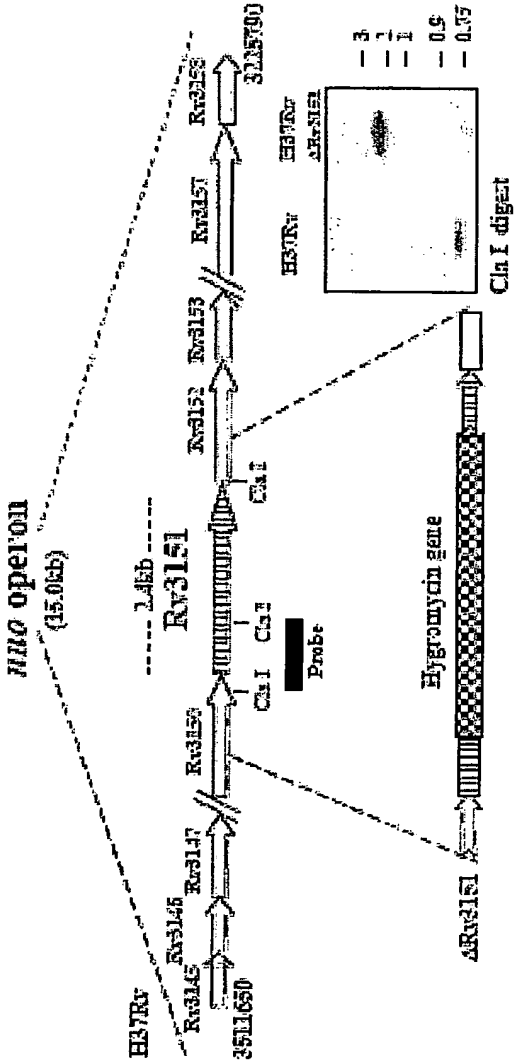

hhoA operon
(15.0kb)
1.4kb
Rv3151
Rv3145
Rv3146
Rv3147
Rv3148
Rv3152
Rv3153
Rv3157
Rv3158
3111650
3115790
Cla I
Probe
Hygromycin gene
ΔRv3151
H37Rv
H37Rv ΔRv3151
3
1
0.9
0.75
Cla I digest

FIG. 26

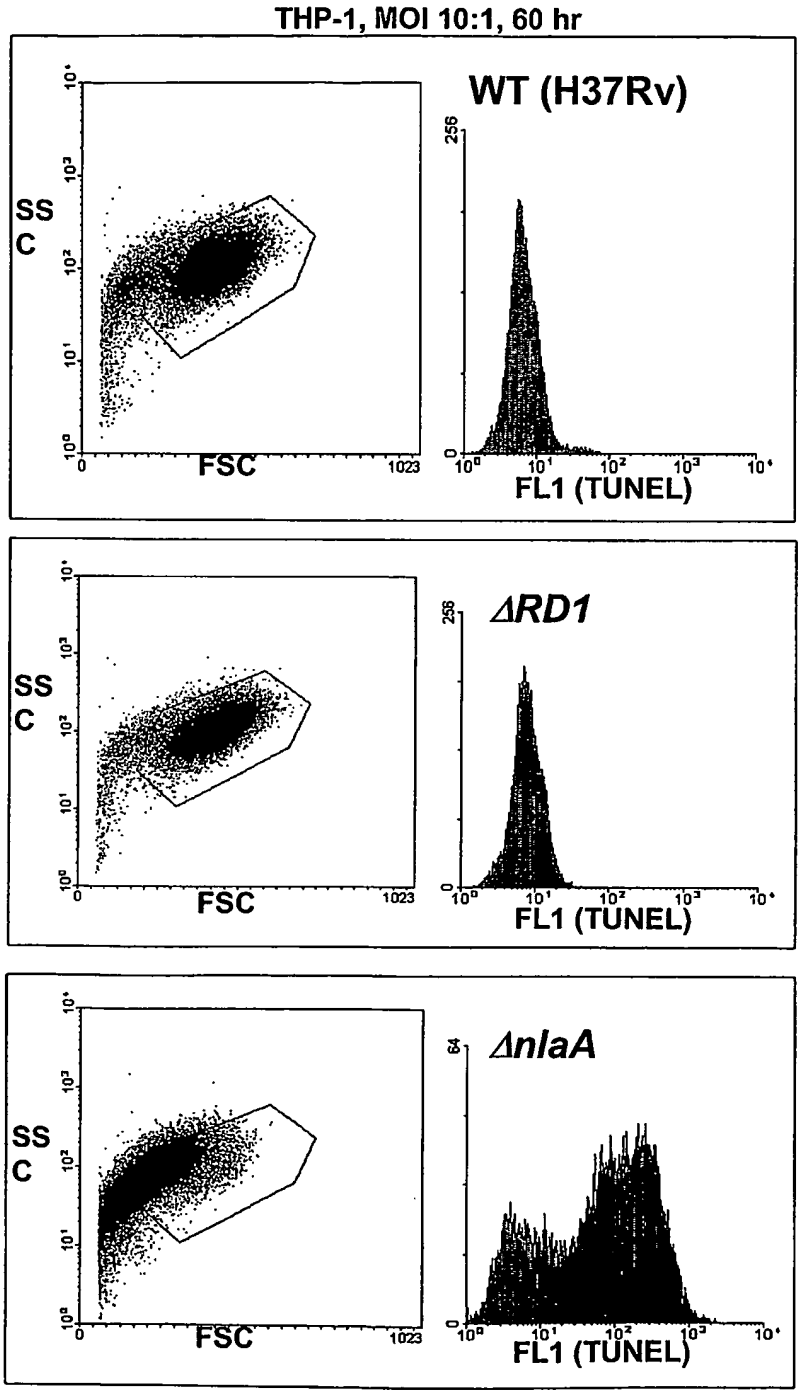

THP-1, MOI 10:1, 60 hr
WT (H37Rv)
ΔRD1
ΔnlaA
SSC
FSC
FL1 (TUNEL)
0
1023
256
64

FIG. 27

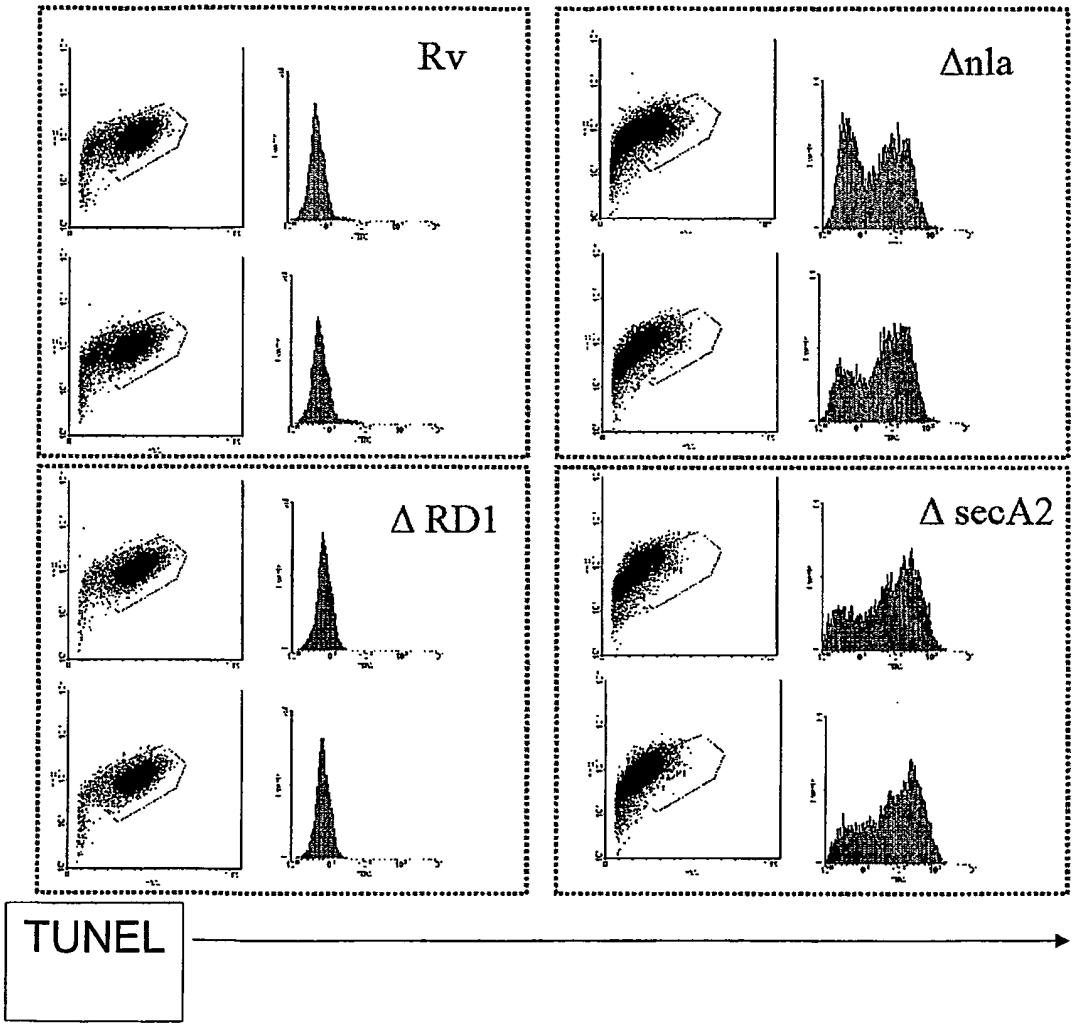


FIG. 28A

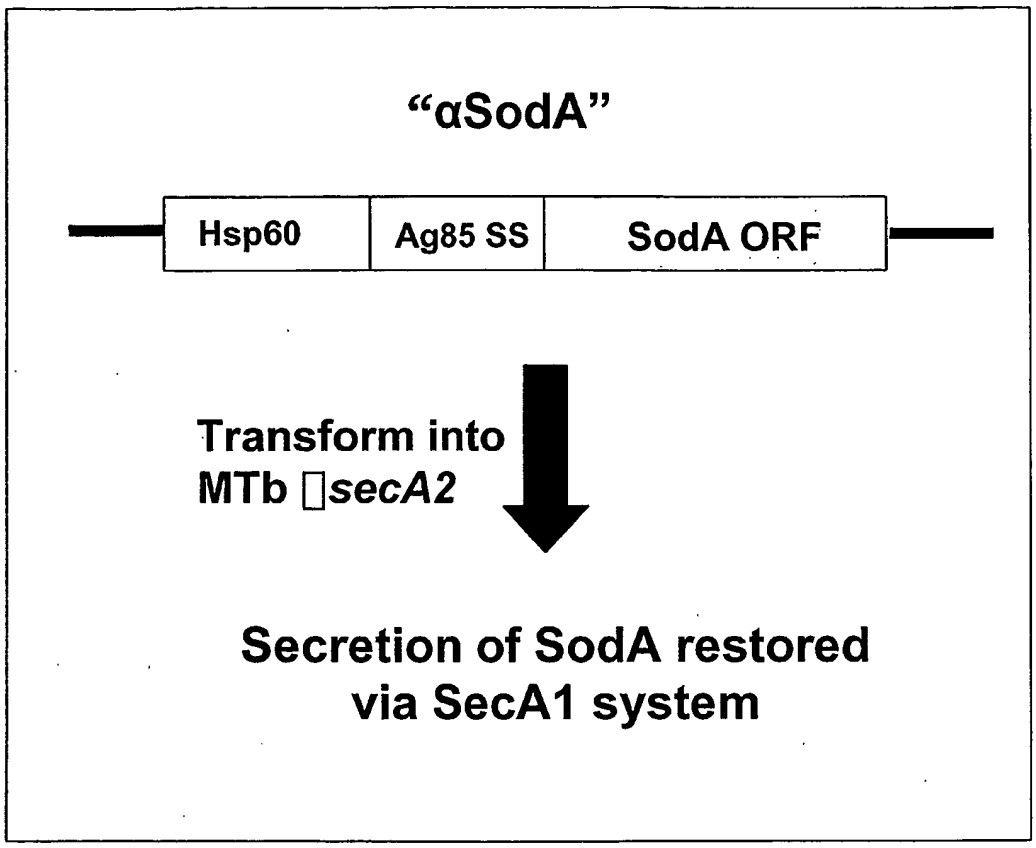

"αSodA"
Hsp60
Ag85 SS
SodA ORF
Transform into
MTb □secA2
Secretion of SodA restored
via SecA1 system

FIG. 28B

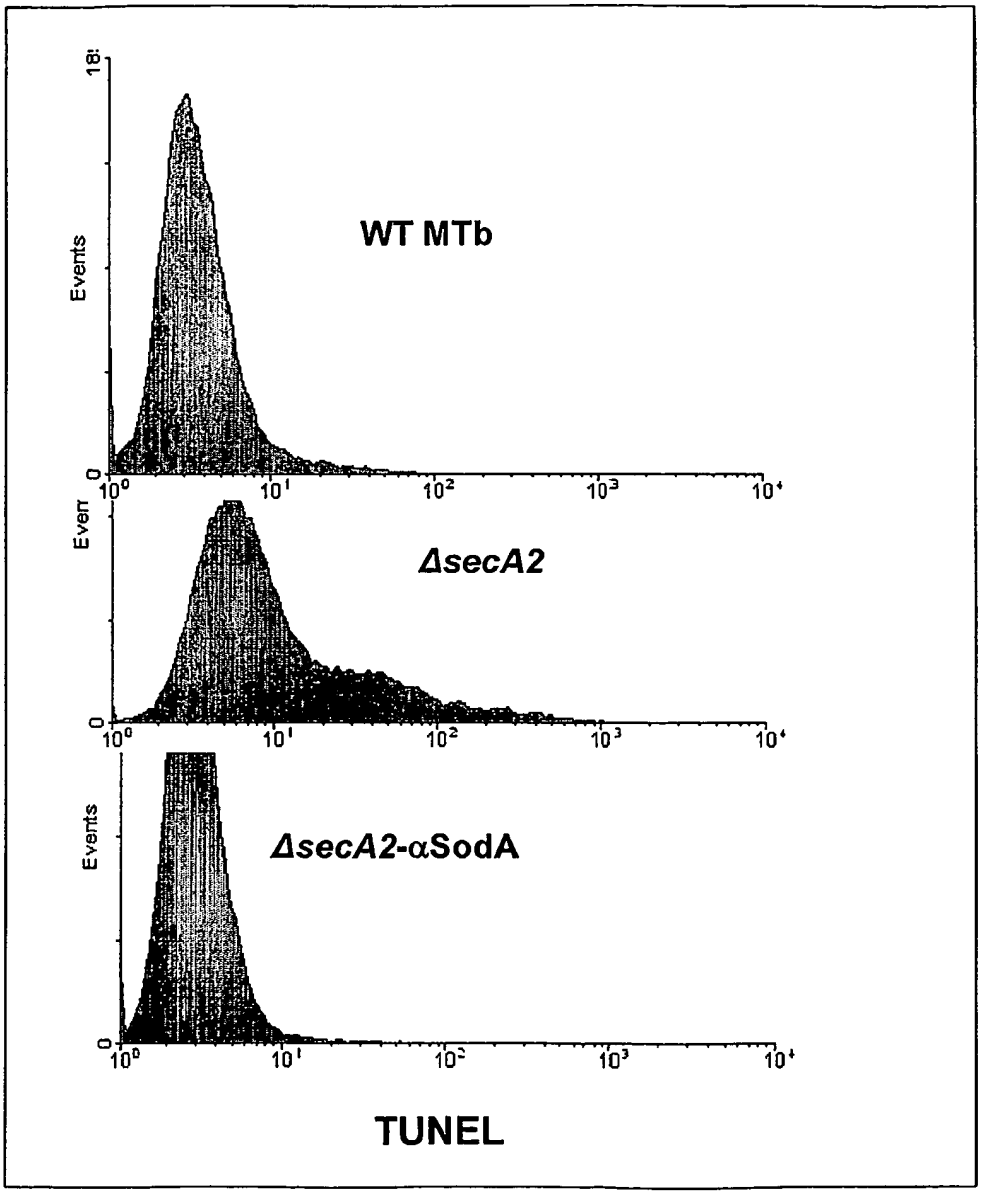
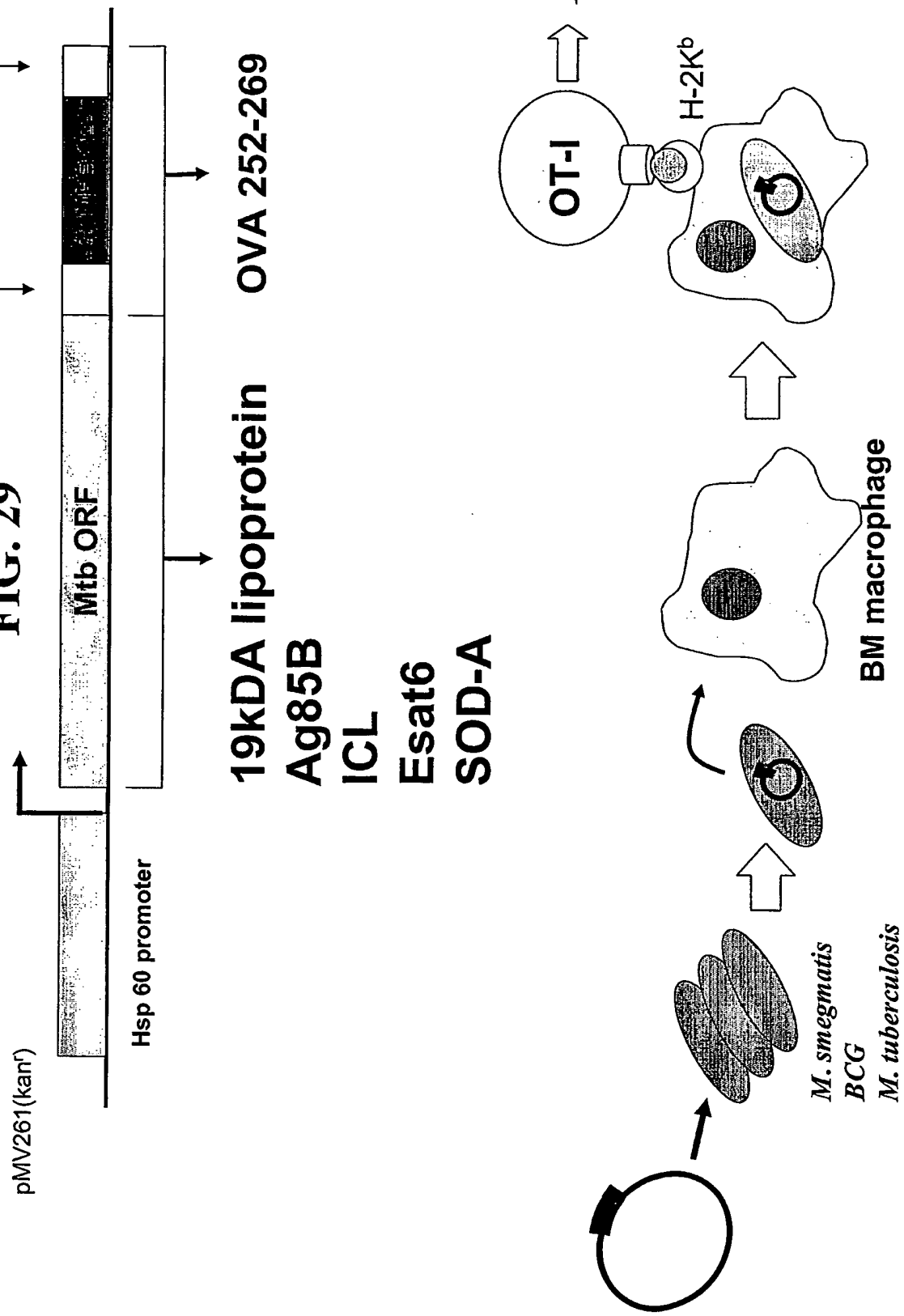


FIG. 29


pMV261(kan^r)
Hsp 60 promoter
5 flanking aa
Mtb ORF
5 flanking aa
19kDA lipoprotein
Ag85B
ICL
Esat6
SOD-A
OVA 252-269
M. smegmatis
BCG
M. tuberculosis
BM macrophage
H-2K^b
OT-I
IFNγ

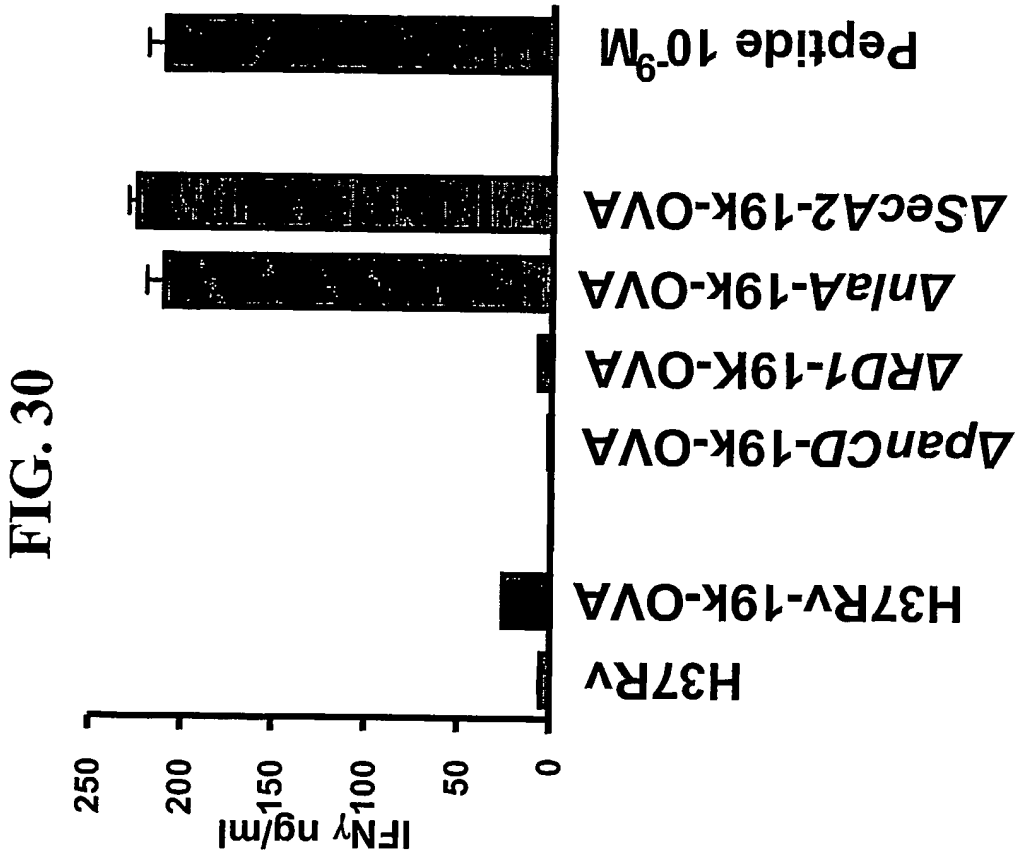

IFNγ ng/ml
250
200
150
100
50
0
H37Rv
H37Rv-19k-OVA
ΔpanCD-19k-OVA
ΔRD1-19k-OVA
ΔlipA-19k-OVA
ΔSecA2-19k-OVA
Peptide 10-9M

FIG. 30

FIG. 31

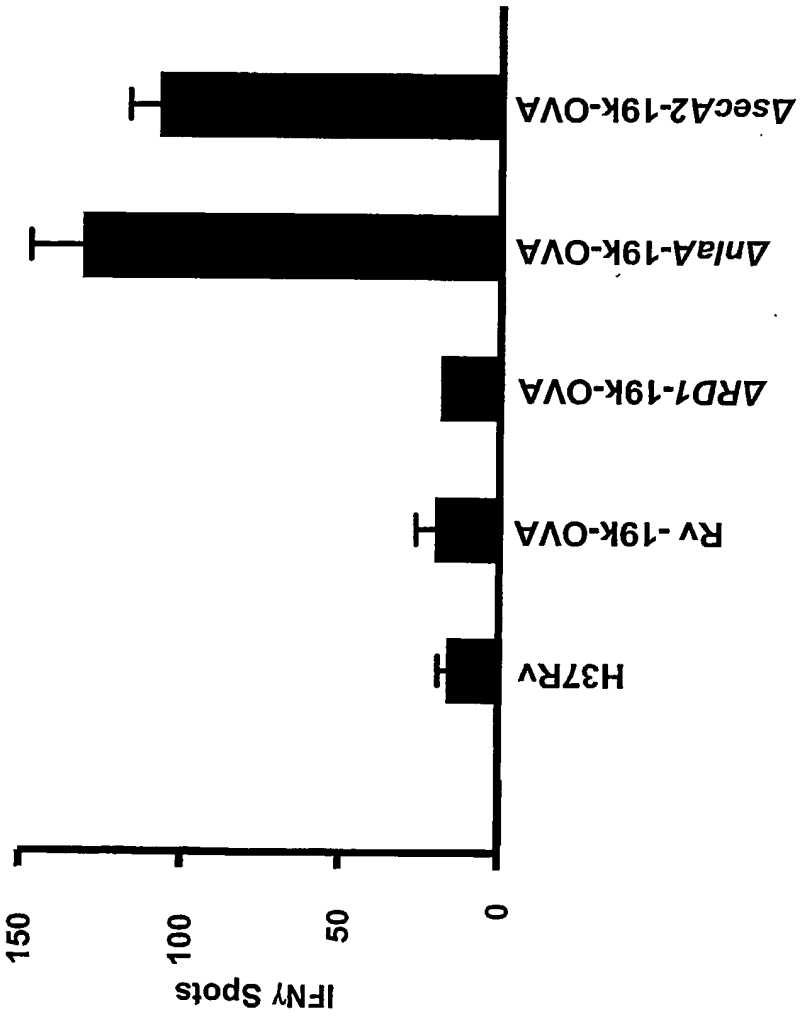

ΔsecA2-19k-OVA
ΔnlaA-19k-OVA
ΔRD1-19k-OVA
Rv -19k-OVA
H37Rv
150
100
50
0
IFNγ Spots

FIG. 32

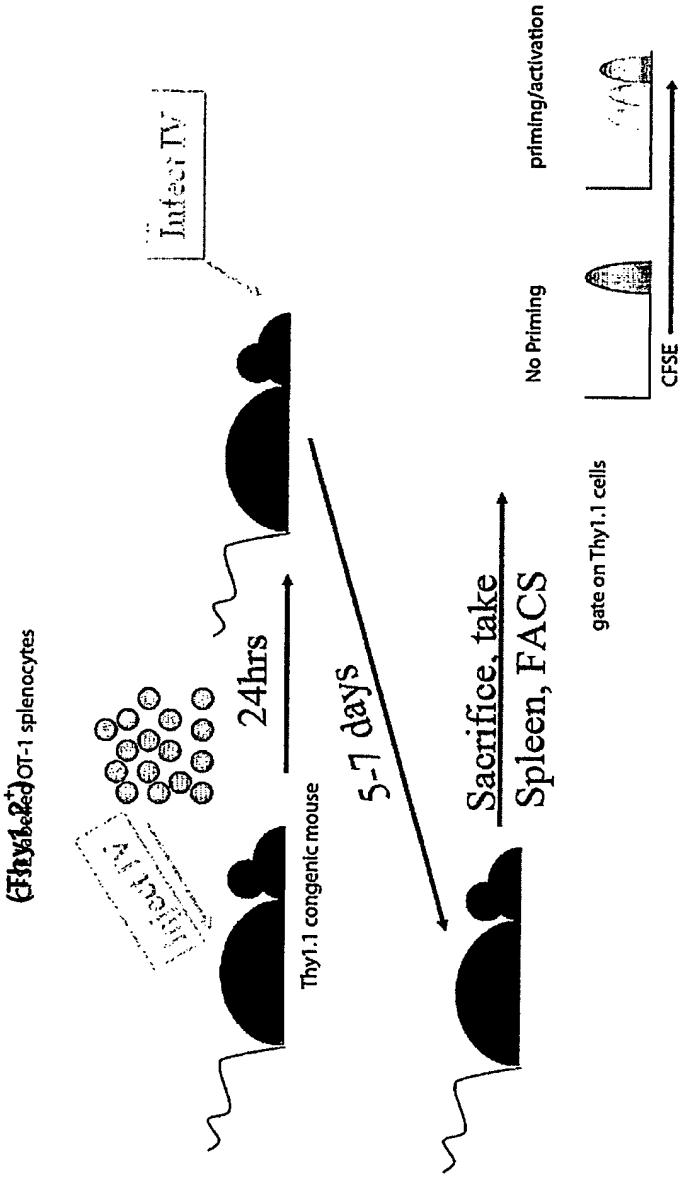

Infect IV
(Thy1.1+) OT-1 splenocytes
24hrs
Thy1.1 congenic mouse
5-7 days
Sacrifice, take
Spleen, FACS
gate on Thy1.1 cells
No Priming
priming/activation
CFSE

FIG. 33

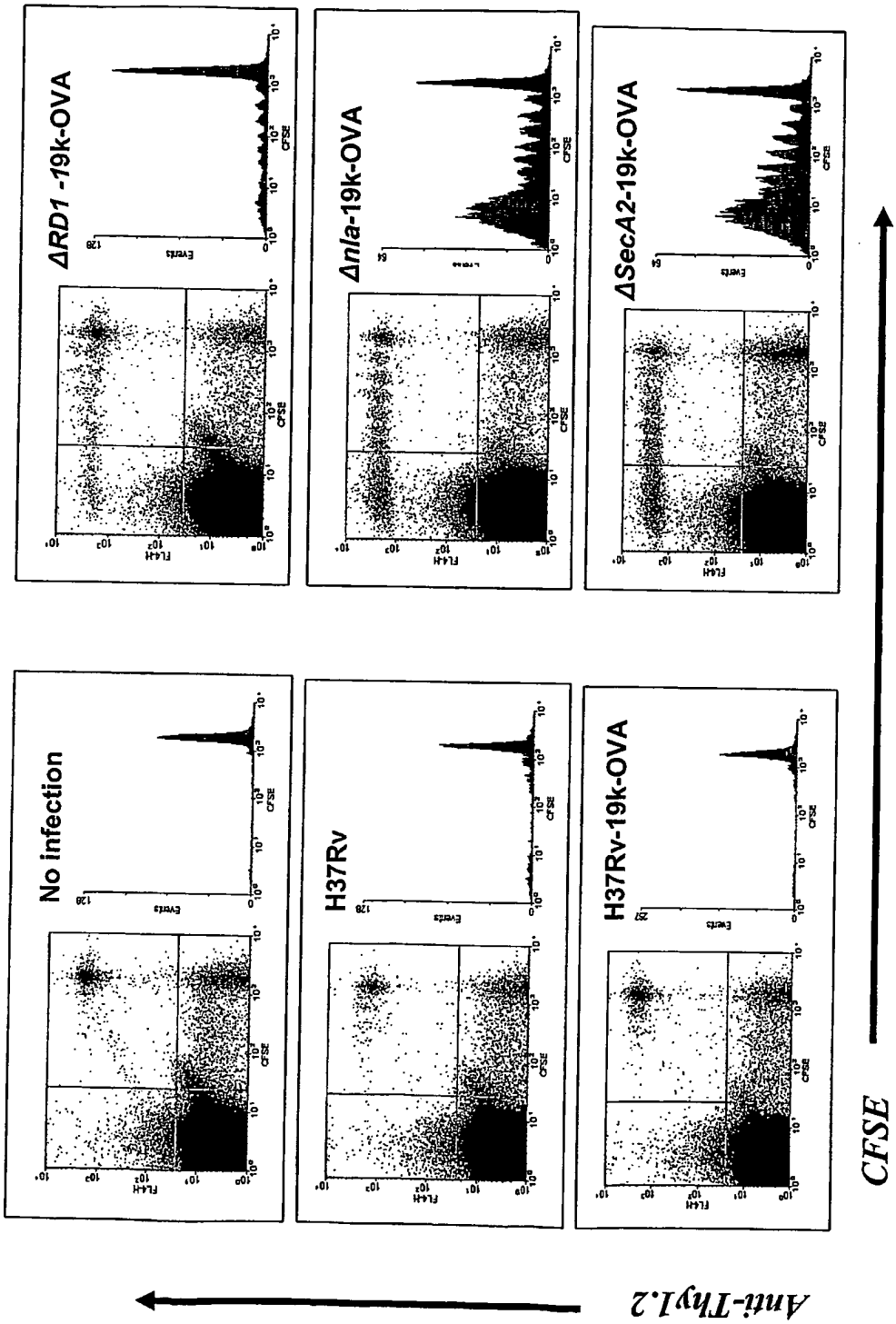
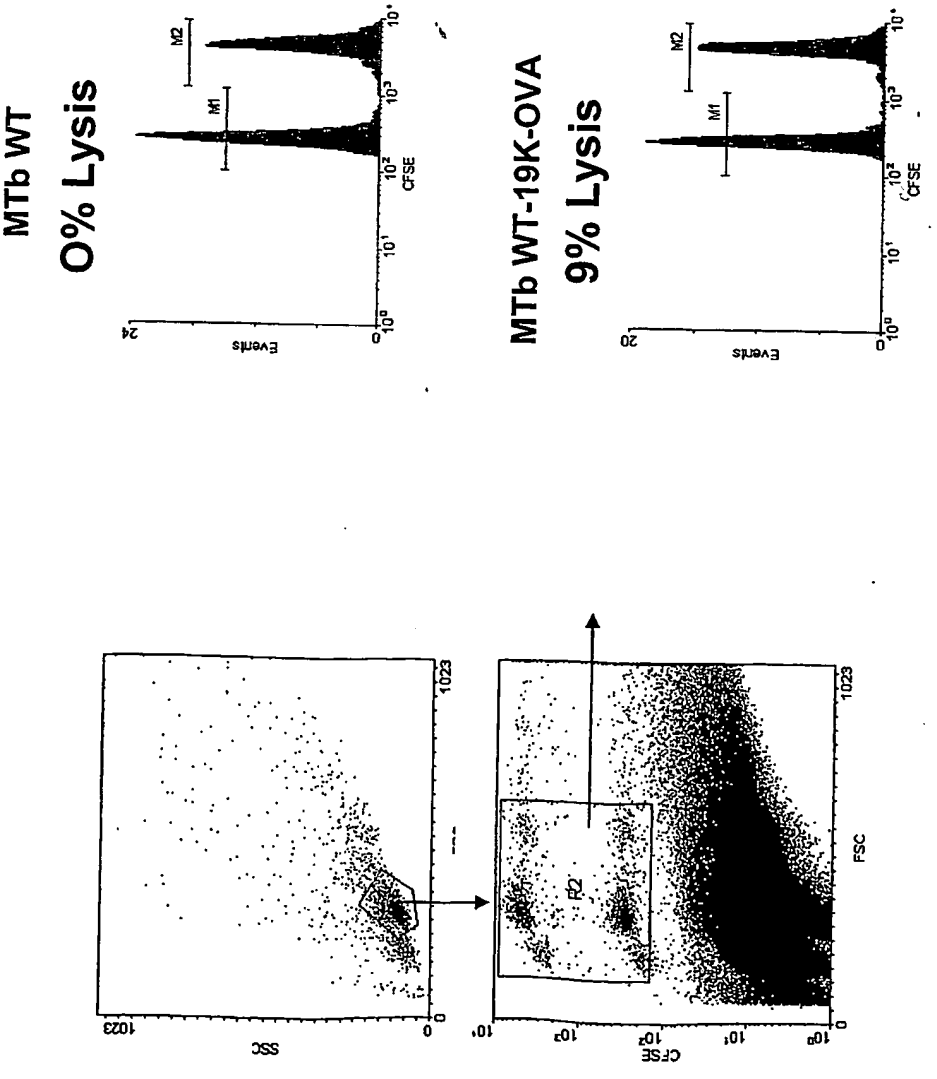

No infection
H37Rv
H37Rv-19k-OVA
ΔRD1 -19k-OVA
Δnla-19k-OVA
ΔSecA2-19k-OVA
Anti-Thy1.2
CFSE

FIG. 35


MTb WT
0% Lysis
MTb WT-19K-OVA
9% Lysis
ΔsecA2-19K-OVA
66% Lysis
ΔnlaA-19K-OVA
69% Lysis
CFSE
Events
SSC
FSC
R2
M1
M2